CELL ADHESION-DEPENDENT COFILIN SERINE 3 PHOSPHORYLATION BY THE INTEGRIN-LINKED KINASE/c-SRC COMPLEX

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Running head: ILK/c-Src complex-mediated cofilin phosphorylation

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Integrin-Linked Kinase (ILK) is involved in signal transduction by integrin-mediated cell adhesion that leads to dynamic actin reorganization. Actin (de)polymerization is regulated by cofilin, whose Ser3 phosphorylation (pS[3]cofilin) inhibits its actin-severing activity. To determine how ILK regulates pS[3]cofilin, we examined the effects of ILK on pS[3]cofilin using normal rat intestinal epithelial (RIE1) cells. Compared to suspended cells, fibronectin (Fn)-adherent cells showed an enhanced pS[3]cofilin, depending on ILK expression and c-Src activity. The ILK-mediated pS[3]cofilin in RIE1 cells did not involve ROCKs, LIMKs, or testicular protein kinases (TESKs), which are known to be upstream of cofilin. The kinase domain of ILK, including proline-rich regions, appeared to interact physically with the SH3 domain of c-Src. *In vitro* kinase assay revealed that ILK immunoprecipitates phosphorylated the recombinant GST-cofilin, which was abolished by c-Src inhibition. Interestingly, EGF treatment abolished the ILK-effects, indicating that the linkage from ILK to cofilin is biologically responsive to extracellular cues. Altogether, this study evidences a new signaling connection from ILK to cofilin for dynamic actin polymerization during cell adhesion, depending on the activity of ILK-associated c-Src.

Integrin-mediated interaction with the extracellular matrix (ECM) triggers intracellular signal cascades that regulate the activity and localization of numerous signaling molecules that consequently modulate diverse cellular functions including actin reorganization (1,2). The recruitment of adaptor and signaling molecules to the cytoplasmic tails of integrin subunits occurs at focal adhesions (FAs) and allows activation of diverse signaling pathways during cell adhesion (3). Integrin-mediated cell adhesion activates FAK, c-Src family kinase, Erk, Akt, and Rho GTPase family (4). The Rho GTPase family mediates actin reorganization via their downstream effectors, including ROCKs, MLCK, LIMKs, and cofilin (5). The actin polymerization/depolymerization status depends on the Ser3 phosphorylation level of cofilin, an actin severing protein (6). Cofilin severs actin filaments when it is dephosphorylated by Slingshot phosphatase or Chronophin, whereas its severing activity can be inhibited when phosphorylated by LIMKs or testicular protein kinase (TESKs) (7).

ILK is a Ser/Thr kinase located at FAs by binding to the β1 integrin cytoplasmic tail, whose overexpression inhibits intestinal epithelial cell (IEC) adhesion to integrin substrates (8). ILK expression was suppressed epigenetically upon adhesion of gastric carcinoma cells, and ILK overexpression reduced adhesion abilities and enhanced anchorage-independent growth (9). In contrast,

ILK expression also correlated with an increased adhesion of prostate cancer cells (10), and ILK was transiently activated upon adhesion of IEC to fibronectin (11). Although the roles of ILK in cell adhesion-mediated actin reorganization have thus been elucidated, depending on the cell type or signaling contexts, mechanistic roles of ILK in the regulation of cofilin phosphorylation remain largely unknown.

In this study, we revealed a new functional linkage of ILK to cofilin, by showing that ILK-associated c-Src affected ILK activity to phosphorylate cofilin during normal epithelial RIE1 cell adhesion.

EXPERIMENTAL PROCEDURES

Cells: The integrin α5-expressing normal rat intestinal epithelial cell line (RIE1-α5) was previously described (12).

Cell conditions, treatments, and immunoblots: Cells were transfected with the indicated plasmid (see below), or siRNA against ILK (siILK, Darmacon) or LIMK1 (siLIMK1, Bioneer Corp., Daejeon, Korea) for 24 h by using Magnetofection™ system (polyMag, OZ Biosciences, France) following the manufacturer's protocols and/or infected with control or ILK-encoding adenovirus (kind gifts from Dr. Kim HS, Seoul National Univ. Hospital, Seoul, Korea) for 24 h. The plasmid to transfect was either pcDNA3-ILK wildtype (WT), E359K or S343A mutant, kinase-dead ROCK1 (DA-1A, a kind gift from Dr. S. Narumiya, Kyoto Univ., Japan), kinase-dead TESK1 (D170A), or TESK2 (D176A) (13), kinase-inactive Y416F c-Src mutant, or kinase-active Y527F c-Src mutant (14). ILK constructs were prepared from ILK wildtype (a kind gift from Dr. R. Juliano, Univ. of North Carolina, Chapel Hill, NC) via polymerase chain reactions (GeneAll, Seoul) and their sequences were confirmed by direct sequence analysis. The cells were then harvested or treated with DMSO or pharmacological inhibitors for 30 min before being kept in suspension or replated on fibronectin (Fn, 10 μg/ml, Chemicon) or poly-Lysine (PL, 10 μg/ml, Sigma)-precoated dishes for 1 h. The inhibitors include LY294002 (20 μM, LC Laboratories), PP2 (10 μM, A.G. Scientific, Inc.), PP3 (10 μM, A.G. Scientific, Inc.), U0126 (40 μM, LC Laboratories), p18 peptide (5 μM, Calbiochem), and Y27632 (15 μM, Calbiochem). In some cases, EGF (50 ng/ml) was treated for the last 5 min of the replating time. Whole cell lysates were prepared by using a modified RIPA buffer (50 mM HEPES, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM NaF, 1 mM Na_2VO_4, 1 mM nitrophenylphosphate, 1 mM PMSF, 10 μg/ml aprotinin, 1 μM pepstatin A, and 10 μM leupeptin), and used in immunoblots using pY^{397}FAK, pY^{416}Src, ILK, c-Src (Santa Cruz Biotechnology), Akt, pS^{473}Akt, Erk1/2, pErk1/2, pT^{508}LIMK1, LIMK1, cofilin, pS^{3}cofilin (Cell Signaling Technology), FAK, GST, ROCK1, α-tubulin (BD Transduction Laboratory), and pSer/Thr (Abcam, Cambridge, UK) antibody.

Immunofluorescence microscopy: Cells on Fn-precoated coverglasses were manipulated with or without pretreatment of PP2 (10 μM, a c-Src family kinase inhibitor) or treatment of EGF (50 ng/ml), as above, or alternatively transfected with pEGFP-ILK. Immunofluorescence for ILK or pY^{416}c-Src (Santa Cruz Biotech.) or staining of actin using Rhodamine-conjugated phalloidin was performed using confocal (MRC-500, Bio-Rad) or fluorescent (BX51, Olympus) microscopy, as described previously (15).

Coimmunoprecipitation: Cells were transfected with inactive c-Src Y416F or kinase-dead ROCK1 (KD-1A) for 48 h. Cell lysates were prepared and immunoprecipitated with anti-ILK antibody, as described previously (15), and probed for c-Src or ILK by immunoblotting.

Expression of fusion proteins and in vitro pull down assay: Diverse human ILK (NP-004508) gene constructs were prepared and cloned into the pGEX4T-3 vector (GE healthcare) at *EcoR*1 and *Xho*I cloning sites. Their sequences were directly confirmed. Each construct, GST-cofilin (a kind gift from Dr. T. Nakamura, Osaka Univ., Japan), or GST-c-Src construct in BL21 *E. coli* was induced by 1 mM IPTG for 4 h. The *E. coli* extracts (with 50 mM Tris-HCl, pH 8.2, 2 mM $MgCl_2$, 0.2 mM $Na_2S_2O_5$, 10% Glycerol, 20% sucrose, 2 mM DTT, 1 mM Na_2VO_4, and protease inhibitors) were then incubated with glutathione-sepharose beads (Amersham Bioscience) for 6 h at 4°C. GST-fusion

proteins bound to beads were washed once with the extracting buffer and 3 times with ice-cold PBS and mixed with RIE1-α5 cell extracts (1 mg protein/condition) for 4 h at 4°C, with or without 10 μM PP2 treatment. After washings as above, proteins were eluted via addition of SDS-PAGE sample buffer and boiling. Eluted proteins were resolved, transferred, and immunoblotted for anti-ILK, GST, or c-Src antibody.

In vitro kinase assay: *In vitro* ILK kinase assay was performed, as described previously (16). ILK immunoprecipitates were prepared overnight using whole cell extracts, mixed with protein A/G beads (Upstate Biotechnology.) for 2 h at 4°C and washed 3 times with ice-cold PBS. An equal amount of ILK immunoprecipitates was then incubated with MBP (3 μg/condition, Sigma) or recombinant GST-cofilin (3 μg/condition) for 30 min at 25°C, in the reaction buffer including 2 μM DTT, 100 μM ATP, 50 mM HEPES, pH 7.0, 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 2 mM NaF. The reactions were stopped with addition of SDS-PAGE sample buffer and they were then used in immunoblots for indicated molecules or ponseu S. staining for MBP.

Statistical analysis: The relative pS^3cofilin under diverse experimental conditions were calculated for graphic presentation (mean ± standard deviation) or for fold difference presentation (with raising fractions not lower than 0.05 to 0.1 for a concise representation) after normalization of pS^3cofilin over cofilin band intensities measured by a densitometry. The Student's *t*-test was performed for comparison of mean values to see if the difference is significant. *p* values < 0.05 were considered significant.

RESULTS

ILK-mediated cofilin phosphorylation on cell adhesion

Although ILK and cofilin are both known to be involved in actin organization, their functional linkage remains largely unknown. While investigating integrin-mediated actin organization, we found that RIE1-α5 cells replated on fibronectin (Fn) showed a higher cofilin Ser3 phosphorylation (pS^3cofilin) than suspended cells did, and exogenous overexpression of ILK further enhanced the adhesion-dependent pS^3cofilin (Fig. 1A). In contrast, cells held in suspension, adherent on poly-lysine, or in a normal serum-containing condition showed no significant pS^3cofilin, even upon ILK overexpression (Fig. 1A). Over time after being suspended or replated on poly-lysine, pS^3colofin was not observed or minor, respectively, whereas cells adherent on Fn showed significant pS^3cofilin levels when ILK was overexpressed. The ILK-enhanced pS^3cofilin was not correlated with the activities of FAK, Akt, or GSK3β (Fig. 1A and data not shown), which are downstream of integrins or of ILK (17); they did not show adhesion- and/or ILK overexpression-dependency, unlike pS^3cofilin levels in Fn-adherent cells overexpressing ILK. However, pY^{118}paxillin was enhanced by cell adhesion, although exogenous ILK did not additionally enhance it (Fig. 1A). The E359K ILK mutant, which is paxillin- and parvin-binding deficient (18) and putatively kinase-dead with a residual kinase activity (19) did not alter the ILK-enhanced pS^3cofilin, whereas the S343A ILK mutant with abolished kinase activity (20) reduced the ILK effects (Fig. 1C). However, cell adhesion-dependent pY^{397}FAK was not altered by expression of different ILK constructs, indicating again that the active ILK could enhance pS^3cofilin in a FAK-independent manner (Fig. 1C). Being consistent with actin polymerization supported by Ser3-phosphorylated cofilin (7), the GFP-ILK transfected cells formed more stress fibers, compared to neighboring untransfected cells (Fig. 1D).

ILK-mediated pS^3cofilin depends on c-Src activity

Next we examined the molecular basis of the ILK effects on cell adhesion. To address this, ILK-infected cells were suspended or replated on Fn for 1 h with or without pretreatment of diverse pharmacological inhibitors. Pretreatment with inhibitors including LY294002 (a specific inhibitor against PI3K and thus its downstream Akt), PP2 (a selective c-Src family kinase inhibitor), U0126 (a specific inhibitor against MEKs and thus its downstream Erk1/2), p18 peptide (a selective

MLCK inhibitor), or Y27632 (a selective ROCK inhibitor) had been done 30 min before cells were held in suspension or replated on Fn. Immunoblots of whole cell lysates from these treatments showed that inhibition of c-Src (lane 9) or ROCK (lane 12) abolished the ILK-enhanced pS^3cofilin (Fig. 2A). Furthermore, pS^3cofilin levels in Fn-adherent cells with diverse treatments were correlated with pY^{416}c-Src levels, but not with pY^{397}FAK or pS^{473}Akt levels (Fig. 2A). Therefore, these data indicate that c-Src activity might be important for pS^3cofilin in the RIE1-α5 cells. Although it is known that ROCK1 phosphorylates LIMKs, which in turn phosphorylate cofilin (21,22), pT^{508}LIMK1 levels under these treatments were not correlated with the pS^3cofilin levels (Fig. 2A). Suppression of LIMK1 by its siRNA transfection nearly abolished the adhesion-mediated pS^3cofilin in control virus-infected cells, but not in ILK overexpression-enhanced pS^3cofilin of Fn-adherent cells (Fig. 2B), indicating that LIMK1 does not play a significant role in the ILK overexpression-enhanced pS^3cofilin. When kinase-dead ROCK1 (KD-1A) was expressed, the basal cell adhesion-dependent pS^3cofilin was completely abolished but ILK overexpression-mediated pS^3cofilin was still significantly maintained (Fig. 2C), indicating that ILK overexpression-mediated pS^3cofilin might be in part independent on ROCK1. However, the siRNA against ILK completely abolished the ILK effects (Fig. 2C). Cofilin is also phosphorylated by the Ser/Thr kinase testicular protein kinase, TESKs (13). Therefore, we evaluated whether kinase-inactive TESK1 (D170A) and TESK2 (D176A) could block the ILK effects. Transfection of the TESK mutants abolished the basal, but not ILK overexpression-enhanced, pS^3cofilin of Fn-adherent cells (Fig. 2D). These observations indicate that ILK enhanced pS^3cofilin in a TESKs-independent manner. Pharmacological inhibition of c-Src by PP2 inhibited the ILK effect on pS^3cofilin (Fig. 2A). Inhibition of ROCK by Y27632 decreased pY^{416}c-Src, as did c-Src inhibitor PP2 (Fig. 2A). Therefore, it is likely that the ROCK inhibition-mediated effect might be a result of nonspecific c-Src inhibition. To confirm that c-Src was required for the ILK effect, we examined pS^3cofilin in suspended or Fn-adherent c-Src/Fyn/Yes-negative ($SYF^{-/-}$) fibroblasts after Ad-ILK infection or Y27632 pretreatment. These conditions did not significantly alter pS^3cofilin, supporting that c-Src was required for the ILK effects (Fig. 2E). Meanwhile, the $SYF^{-/-}$ cells appeared to regulate pS^3cofilin via LIMK1, since LIMK1 suppression reduced pS^3cofilin (Fig. 2F).

ILK binds c-Src

Since ILK increased pS^3cofilin in a c-Src activity-dependent manner, we examined whether ILK might bind active c-Src (i.e., pY^{416}c-Src). We did this by microscopic visualization of their colocalization in Fn-adherent cells using a specific c-Src inhibitor, PP2. ILK was colocalized with pY^{416}c-Src on the periphery of Fn-adherent cells, and PP2 pretreatment abolished their colocalization (Fig. 3A). We next performed an *in vitro* pull-down assay to determine if ILK directly interacts with c-Src using recombinant GST-c-Src proteins and RIE1-α5 extracts. ILK from only Fn-adherent (but not suspended) cells bound to the GST-c-Src wildtype, which was abolished by PP2 treatment (Fig. 3B, left). This observation indicates that the binding depended on c-Src activity. c-Src has a SH2 and a SH3 domain at its N-terminal end (23). The recombinant GST-SH3 (of c-Src), but not GST alone, showed significant binding to ILK from Fn-adherent (but not suspended) cells, although GST-SH2 (of c-Src) showed just a detectable binding (Fig. 3B, right). Next we determined which part of ILK binds to c-Src. Since ILK bound to GST-SH3 (of c-Src), the proline-rich regions of ILK could interface with the c-Src SH3 domain. Human ILK (gene number; NP-004508) has 3 proline-rich regions in its kinase domain. The *in vitro* pull-down assay of the recombinant GST-ILK proteins (Fig. 3C) clearly showed a direct interaction between the intact kinase domain of ILK and c-Src; GST-ILK proteins without whole or any part of the kinase domain of ILK failed to bind c-Src, whereas GST-ILK proteins with wildtype or the intact kinase domain itself of ILK bound to c-Src (Fig. 3D). In addition, coimmunoprecipitation of c-Src was next examined from ILK immunoprecipitates of RIE1-α5 extracts. ILK clearly coimmunoprecipitated c-Src in cells adherent on Fn, but not suspended, and the association was

abolished by inactive Y416F c-Src expression (Fig. 3E). These observations indicate that ILK can associate with active c-Src. However, kinase-dead ROCK1 expression did not completely abolish the cell adhesion-dependent ILK association with c-Src, indicating again that the ILK/c-Src complex formation is at least in part irrelevant to the ROCK1 activity (Fig. 3E).

Active c-Src-associated ILK phosphorylated cofilin

ILK-enhanced pS^3cofilin depended on c-Src activity, since pretreatment of PP2 to inhibit c-Src family kinase abolished ILK-enhanced pS^3cofilin whereas treatment of PP3 (a negative control compound of PP2) did not (Fig. 4A and 2A). In addition, ILK bound directly to c-Src (Fig. 3). We thus examined if ILK kinase activity might be modulated via c-Src association, by performing an *in vitro* kinase assay of ILK immunoprecipitates with using myelin basic protein (MBP) or GST-cofilin as substrates. ILK immunoprecipitates from adherent (but not suspended) cells efficiently phosphorylated Ser/Thr residues of MBP (Fig. 4B) and Ser3 of recombinant GST-cofilin (Fig. 4C), which were abolished by PP2 treatment into the reaction mixture (Fig. 4B and 4C). In addition, ILK immunoprecipitates from adherent cells had a higher pY^{416}c-Src, compared to those from suspended cells, which was inhibited by PP2 treatment (Fig. 4C). These observations suggest that active c-Src-associated-ILK caused pS^3cofilin. Since interaction of ILK with GST-SH2 (of c-Src) was just detectable (Fig. 3B, right), it is likely that the ILK-associated c-Src might phosphorylate and affect the ILK activity. However, we could not observe Tyr phosphorylation in ILK (data not shown), indicating that active c-Src might activate ILK indirectly through unidentified molecule(s).

EGF treatment blocked the ILK-enhanced pS^3cofilin

We next evaluated if extracellular stimulation by EGF affected the adhesion-dependent ILK effects. EGF treatment of cells replated on Fn was performed for the last 5 min of the replating period, before cell harvests and immunoblots. EGF treatment abolished the basal and ILK overexpression-enhanced pS^3cofilin in Fn-adherent cells (Fig. 5A). EGF treatment and/or inactive Y416F c-Src expression abolished the basal and ILK-enhanced pS^3cofilin in Fn-adherent cells, although EGF-mediated Erks activation in the adherent cells was irrelevant to ILK infection (Fig. 5A and B). Expression of Y527F c-Src (where the inhibitory phosphorylation site was mutated) did not alter pS^3cofilin levels, compared to the control vector transfected cells (Fig. 5C), indicating that cell adhesion-mediated activation of endogenous c-Src could afford ILK/cofilin in the absence of EGF treatment and EGFR/Erk pathway in the presence of EGF treatment (see discussion). EGF treatment of adherent cells abolished the colocalization between ILK and pY^{416}c-Src (Fig. 5D), ILK binding to GST-c-Src WT or -SH3 (of c-Src) (Fig. 3B), and ILK activity for MBP substrate (Fig. 4B). Therefore, the EGF treatment data indicate that the linkage from ILK to cofilin is biologically functional.

DISCUSSION

Integrin-mediated cell adhesion triggers diverse intracellular signals leading to actin polymerization. ILK binds to integrin β subunits by forming a modular platform for protein complexes to rearrange actin filaments (24). Meanwhile, cofilin severs actin filaments, depending on its Ser3 phosphorylation status (7,25). Although ILK and cofilin are both involved in the actin organization, their functional linkage is largely unknown. Our observations in this study provide evidence for a new functional linkage of ILK to cofilin for actin polymerization, showing that association between ILK and c-Src affected ILK activity to phosphorylate cofilin during cell adhesion.

Although integrin-mediated cell adhesion is suggested to involve ILK/paxillin/FAK complexes at the FAs (26), ILK-enhanced pS^3cofilin was irrelevant to FAK, paxillin, and Akt phosphorylation. The E359K ILK mutant deficient for paxillin- and parvin-binding (18) and putatively kinase-dead with a residual kinase activity (19) still supported the ILK-enhanced pS^3cofilin, but the S343A ILK mutant with abolished kinase activity (20) blocked the ILK-mediated effects. Therefore, the ILK effect on cofilin phosphorylation appeared to require its activity.

Cofilin is known to be phosphorylated by LIMKs or TESKs (13,22). Furthermore, ROCK1 phosphorylates LIMKs (21). Interestingly, the adhesion-dependent pS^3cofilin in RIE1-α5 cells without exogenous ILK expression in this study could be regulated by ROCK1, LIMK1, TESKs, and ILK as well; transient transfection of kinase-dead ROCK1 or TESKs or of siLIMK1 or siILK abolished cell-adhesion-dependent pS^3cofilin. However, our results indicate that ILK overexpression-mediated pS^3cofilin did not significantly involve ROCK1, LIMK1, and TESKs. The basal cell adhesion-dependent pS^3cofilin was abolished by expression of kinase-dead ROCK1 but ILK overexpression-mediated pS^3cofilin was still significantly maintained. Kinase-dead ROCK1 expression did not disrupt the association between ILK and c-Src. It is thus likely that the ILK affects pS^3cofilin, at least partially, in a ROCK1-independent manner. In addition, pT^{508}LIMK1 was not correlated with the ILK effects and furthermore LIMK1 suppression did not significantly inhibit the ILK overexpression-mediated pS^3cofilin. These observations indicate that ILK overexpression-mediated pS^3cofilin in RIE1-α5 cells might be irrelevant to LIMK1. Meanwhile, α-parvin (actopaxin) binds ILK, paxillin (27) and TESK1 to cause cellular spreading on Fn (28), indicating that ILK can be relevant to TESKs. However, transfection of the kinase inactive TESK1 and 2 mutants abolished the basal, but not ILK overexpression-enhanced, pS^3cofilin of Fn-adherent cells, indicating that ILK enhanced pS^3cofilin in a TESKs-independent manner. However, the ILK overexpression-mediated pS^3cofilin appeared to be dependent on c-Src activity in adherent cells; pharmacological inhibitor treatment or kinase-inactive c-Src transfection abolished ILK-mediated pS^3cofilin in Fn-adherent cells. On the other hand, pS^3cofilin in c-Src-deficient $SYF^{-/-}$ cells was not dependent on ILK (-overexpression), but required LIMK1. Therefore, these observations suggest that active c-Src may be involved in the ILK-mediated pS^3cofilin.

ILK binds diverse molecules to rearrange actin filaments during integrin-mediated cell adhesion (26), although the interaction of ILK with c-Src or cofilin remains relatively unknown. Consistent with the observations that ILK enhanced pS^3cofilin in a c-Src activity dependent manner, *in vitro* pull-down analysis revealed that the kinase domain of ILK interacted directly with the SH3 domain of c-Src. pY^{416}c-Src of ILK immunoprecipitates from adherent cells was observed higher than that from suspended cells. Furthermore, *in vitro* kinase assays also supported that ILK could interact with and phosphorylate GST-cofilin, which was abolished by inhibition of c-Src that was presumably associated with ILK. Although the kinase domain of ILK is involved in many protein interactions for actin reorganization (19), the interaction of ILK with c-Src is for the first time revealed and indicates a new signaling linkage to regulate cofilin phosphorylation and actin polymerization during adhesion of RIE1-α5 cells. The ILK-enhanced pS^3cofilin seems not to be biologically functional or significant in the signal contexts that c-Src is not significantly activated or regulated, however, since we observed that pS^3cofilin in c-Src/Fyn/Yes-deficient $SYF^{-/-}$ cells was regulated by LIMK1, but not by ILK/c-Src linkage.

Interestingly, EGF treatment abolished the basal and ILK overexpression-enhanced pS^3cofilin in Fn-adherent RIE1-α5 cells. Signaling cross-talks between the integrin and the growth factor receptor (29) can be regulated by ILK (30). This finding was thus unexpected since EGF treatment is mitogenic and positive for Akt and GSK3β activation (31) and since EGF signaling requires c-Src (32). Previous reports showed that EGF treatment or EGFR activity was negatively or positively correlated with pS^3cofilin, indicating contradictory roles of EGFR signaling in pS^3cofilin (33,34). The observation of no significant change in Erks even with Y416F c-Src expression, which led to blockade of ILK-mediated pS^3cofilin, supports the idea that there can be two distinct pools of active c-Src involved either in ILK-mediated pS^3cofilin or in EGFR/Erks pathway; cell adhesion-mediated activation of endogenous c-Src could support ILK/cofilin in the absence of EGF treatment and EGFR/Erks pathway in the presence of EGF treatment (Fig. 5C). We may thus speculate that EGF treatment-dependent intracellular signal pathways might extract out

active c-Src from ILK/c-Src/cofilin complex or linkage, leading to down-regulation of ILK-mediated Ser3 phosphorylation of cofilin.

In neutrophils, cofilin was dephosphorylated by treatment of diverse chemoattractants via phosphoinositide-specific phospholipase C (PI-PLC) -mediated PKC and Ca^{2+} pathways (35). EGF can act as a chemoattractant for spatial and temporal regulation of actin cytoskeleton via modulation of actin binding protein, such as cofilin. ILK is a Ser/Thr kinase binding to the β1 integrin cytoplasmic tail at focal adhesions (8). With dynamic focal adhesion turnover and membrane ruffling around the leading edge of a migrating cell, integrin-mediated adhesion may lead to activation of ILK to regulate cofilin phosphorylation and activity. EGF treatment caused activation of cofilin to sever actin filaments to result in early generation of free barbed ends that participate in the nucleation of actin polymerization at leading edges of migratory MTLn3 cells (36). Therefore, it may not be ruled out that ILK/c-Src complex-mediated regulation of cofilin phosphorylation and activity participates in severing of actin filaments to generate their free barbed ends at leading edges of RIE1-α5 cells, although regulation of cofilin activity by phospholipid PIP2 was recently shown to occur at the leading edge of carcinoma MTLn3 cells (37). More detailed understanding of the significance of the ILK/c-Src complex-mediated cofilin phosphorylation in dynamic actin remodeling can have important implications for our comprehension of physiological and pathological processes, such as cell adhesion/migration and cancer metastasis.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. ILK-enhanced cofilin Ser3 phosphorylation upon integrin-mediated cell adhesion. (A) RIE1-α5 cells were infected with control (Ad-Cont) or ILK-encoding adenovirus (Ad-ILK) for 24 h (N), trypsinized, washed twice with serum-free media containing 1% BSA, rolled over (60 rpm) for 1 h at 37^{o}C to null-out the basal signaling activity, and then either held in suspension (S), replated on fibronectin (Fn)- or poly-lysine (PL)-precoated dishes for 1 h. (B) Cells were infected with Ad-ILK for 24 h (0), and manipulated to be suspended (Sus) or Fn-adherent (Fn), as in (A), for the indicated periods. (C and D) Cells were transfected with ILK wildtype, E359K or S343A mutant (C) or GFP-ILK (D) for 48 h, and manipulated, as above. Whole cell lysates were prepared and used for immunoblots for the indicated molecules (A to C), or cells on Fn-precoated coverglasses were stained for actin (D). The relative pS^{3}cofilin were calculated for graphic presentation (mean ± standard deviation) after normalization of pS^{3}cofilin over cofilin band intensities measured by a densitometry. * indicates statistical significance (p<0.05). Data shown represent 3 different experiments.

Fig. 2. The ILK effect depends on c-Src activity. (A) Ad-ILK infected RIE1-α5 cells were pretreated with DMSO or pharmacological inhibitors, 30 min before the cell manipulation to be held in suspension (Sus) or replated on fibronectin (Fn), as in Fig. 1A. In the middle of the rolling-over process prior to the

cell manipulation, the DMSO or inhibitor was added directly into the replating media (serum-free media with 1% BSA) at 20 μM for LY294002, 10 μM for PP2, 40 μM for U0126, 5 μM for p18 peptide, or 15 μM for Y27632. (B to D) One day after RIE1-α5 cells were transfected with siRNA against LIMK1 (siLIMK1, B), mock or kinase-dead ROCK1 plasmid (KD-1A, C), or siRNA against ILK (siILK, C) or kinase-dead TESK1 D170A or TESK2 D176A plasmid (D) for 24 h, the cells were infected with control (Ad-Cont, -) or ILK adenovirus (Ad-ILK) for 24 h, before the manipulation of cells, as in Fig. 1A. A reduced extraction of LIMK1 was observed in Fn-adherent cells, presumably due to a less solubilization of LIMK1 upon cell adhesion. (E) $SYF^{-/-}$ fibroblasts infected with Ad-Cont (Cont or Y27632) or Ad-ILK for 48 h were manipulated, without (Cont) or with pretreatment with a specific ROCK inhibitor Y27632 (15 μM), 30 min before the cell manipulation, as in Fig.1A. (F) One day after $SYF^{-/-}$ cells were transfected with siLIMK1, the cells were infected with Ad-Cont or Ad-ILK for additional 24 h, before manipulation of the cells, as above. Whole cell lysates were prepared and immunoblotted for the indicated molecules. The relative pS^3cofilin for diverse experimental conditions were calculated for graphic presentation (mean ± standard deviation) after normalization of pS^3cofilin over cofilin band intensities measured by a densitometry. * and ** indicate statistical significance ($p<0.05$) and insignificance ($p\geq0.05$), respectively. Data shown represent 3 isolated experiments.

Fig. 3. The kinase domain of ILK directly bound to the SH3 domain of c-Src. (A) RIE1-α5 cells replated on Fn-precoated coverglasses for 1 h with or without pretreatment of PP2 (10 μM), as explained in Fig. 2A, were immunostained for ILK and pY^{416}c-Src prior to confocal analysis. Scale bar: 10 μm. (B) RIE1-α5 cell lysates or extracts were prepared from cells manipulated to be suspended (Sus) or replated onto fibronectin (Fn), as in Fig. 1A. EGF (50 ng/ml) was treated for the last 5 min of the replating 1 h incubation. An equal amount of proteins was incubated with recombinant GST or GST-c-Src proteins bound to glutathione-sepharose beads at 4^oC with rolling. After washings with ice-cold PBS (three times), proteins were eluted from the beads before immunoblots for the indicated molecules and ponseu S. staining for MBP. GST-SH2 or -SH3 depicts recombinant GST-fused SH2 or SH3 domain of c-Src, respectively. (C) Recombinant GST-ILK proteins. AR; ankyrin repeats, PH; pleckstrin homology. 'Kin' or 'ase' depicts the N-terminal or C-terminal part of the ILK kinase domain, which include two (PR_{12}) or one (PR_3) proline-rich regions, respectively. (D) Recombinant GST-ILK proteins were incubated with whole cell lysates (WCL) from Fn-adherent RIE1-α5 cells. The pulled-down proteins and cell lysates (WCL) were immunoblotted against GST or c-Src, as explained in Experimental Procedures. * indicates proteolytic products. (E) One day after RIE1-α5 cells were transfected with control (Mock) or inactive Y416F c-Src (Y416F) or kinase-dead ROCK1 (KD-1A) plasmid for 24 h, the cells were infected with Ad-ILK for additional 24 h. The cells were then manipulated to be suspended (Sus) or replated onto Fn (Fn), as above. An equal amount of whole cell lysates (WCL) was immunoprecipitated with anti-ILK antibody, before immunoblots of the ILK immunoprecipitates (IP: ILK) and lysates (WCL) for c-Src and ILK. Data shown represent 3 independent experiments.

Fig. 4. Active c-Src-associated ILK phosphorylated cofilin. (A) Cells infected with either Ad-cont or Ad-ILK for 48 h were pretreated with or without 10 μM PP2 or PP3 (a negative control of PP2) 30 min before the cell manipulation to be suspended (Sus) or replated on fibronectin (Fn), as above. Whole cell lysates from RIE1-α5 cells under diverse conditions were immunoblotted for the indicated molecules. (B and C) RIE1-α5 cells were infected with Ad-Cont (C) or Ad-ILK (+) for 48 h, before the cell manipulation for suspension (Sus) or replating on Fn (Fn) with or without PP2 pretreatment or EGF treatment, as above (B). Alternatively, RIE1-α5 cells were manipulated to be suspended (Sus) or replated on Fn (Fn) with DMSO or PP2 pretreatment, as above (C). The whole cell lysates from the cells were prepared and used in immunoprecipitation with anti-ILK antibody. An equal amount of the ILK immunoprecipitates was mixed with MBP (B) or recombinant GST-cofilin (C), as substrates for *in vitro* kinase assay. The resultant reaction mixtures and lysates were resolved and immunoblotted for the indicated molecules. MBP was stained with ponseu S. and GST-cofilin was immunoblotted with anti-

GST for equal amounts of substrates. The relative pS^3cofilin for diverse experimental conditions were calculated for graphic presentation (mean ± standard deviation) after normalization of pS^3cofilin over cofilin band intensities measured by a densitometry. * and ** indicate statistical significance ($p<0.05$) and insignificance ($p\geq0.05$), respectively. Data shown represent 3 isolate experiments.

Fig. 5. EGF treatment abolished ILK-enhanced pS^3cofilin on cell adhesion. (A) RIE1-α5 cells were infected with either Ad-Cont or Ad-ILK for 48 h and then manipulated to be suspended (Sus) or replated on Fn (Fn) with or without EGF treatment, as above. (B and C) One day after cells were transfected with either control (Mock), inactive Y416F (B and C), or active Y527F (C) c-Src mutant plasmid, the cells were infected with either Ad-Cont or Ad-ILK for additional 24 h before the cell manipulation to be suspended (Sus) and replated on Fn (Fn) with or without EGF treatment, as above. Whole cell lysates were prepared and used in immunoblots for the indicated molecules (A, B, and C). The fold differences in pS^3cofilin for diverse experimental conditions were calculated after normalization of pS^3cofilin over cofilin band intensities measured by a densitometry, with raising fractions not lower than 0.05 to 0.1 for a concise representation. (D) RIE1-α5 cells replated on Fn-precoated coverglasses with or without EGF treatment, as above, were doubly immunostained for ILK and pY^{416}c-Src prior to confocal microscopy analysis. Scale bar is 10 μm. Data shown represent 3 different experiments.

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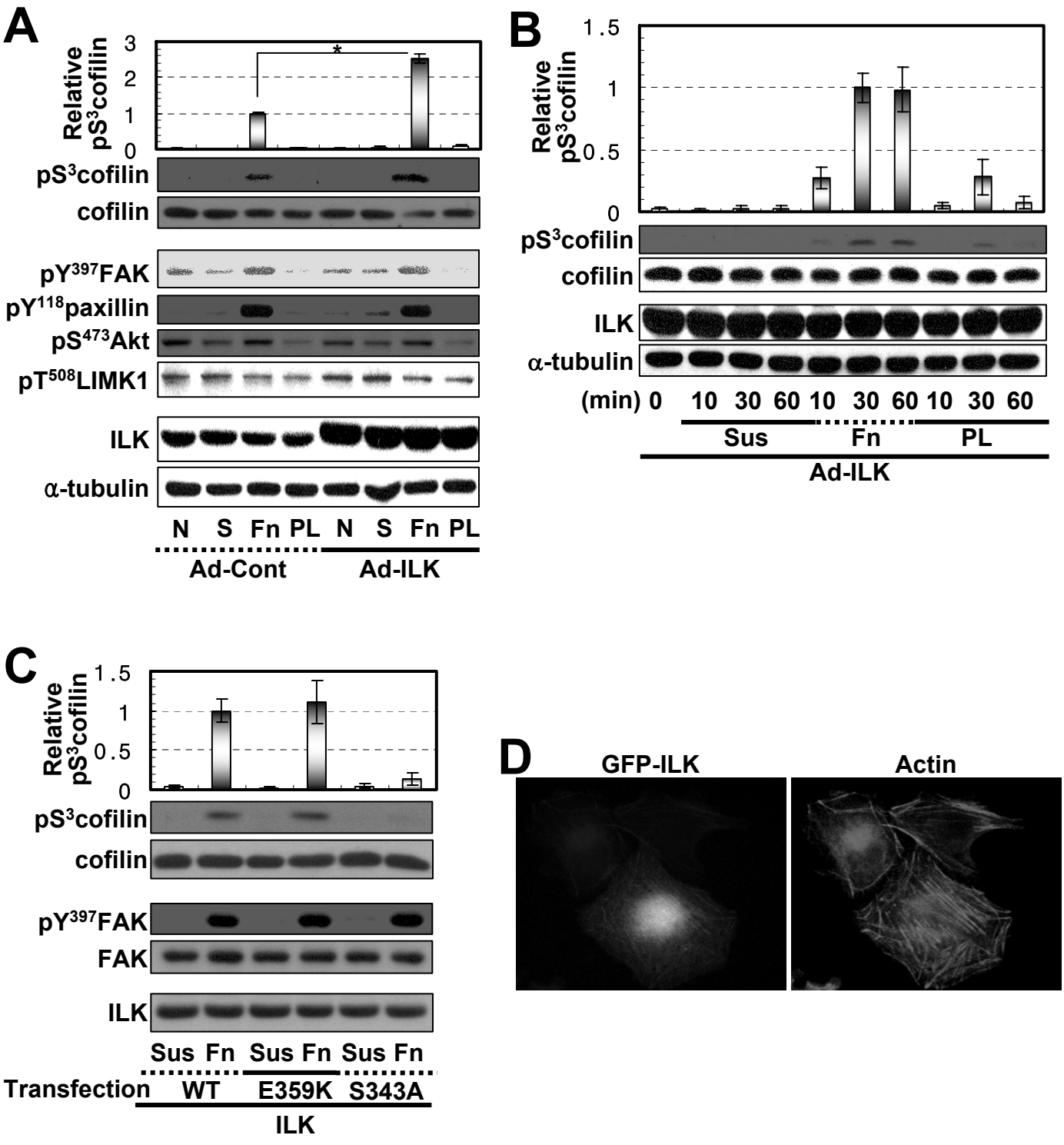

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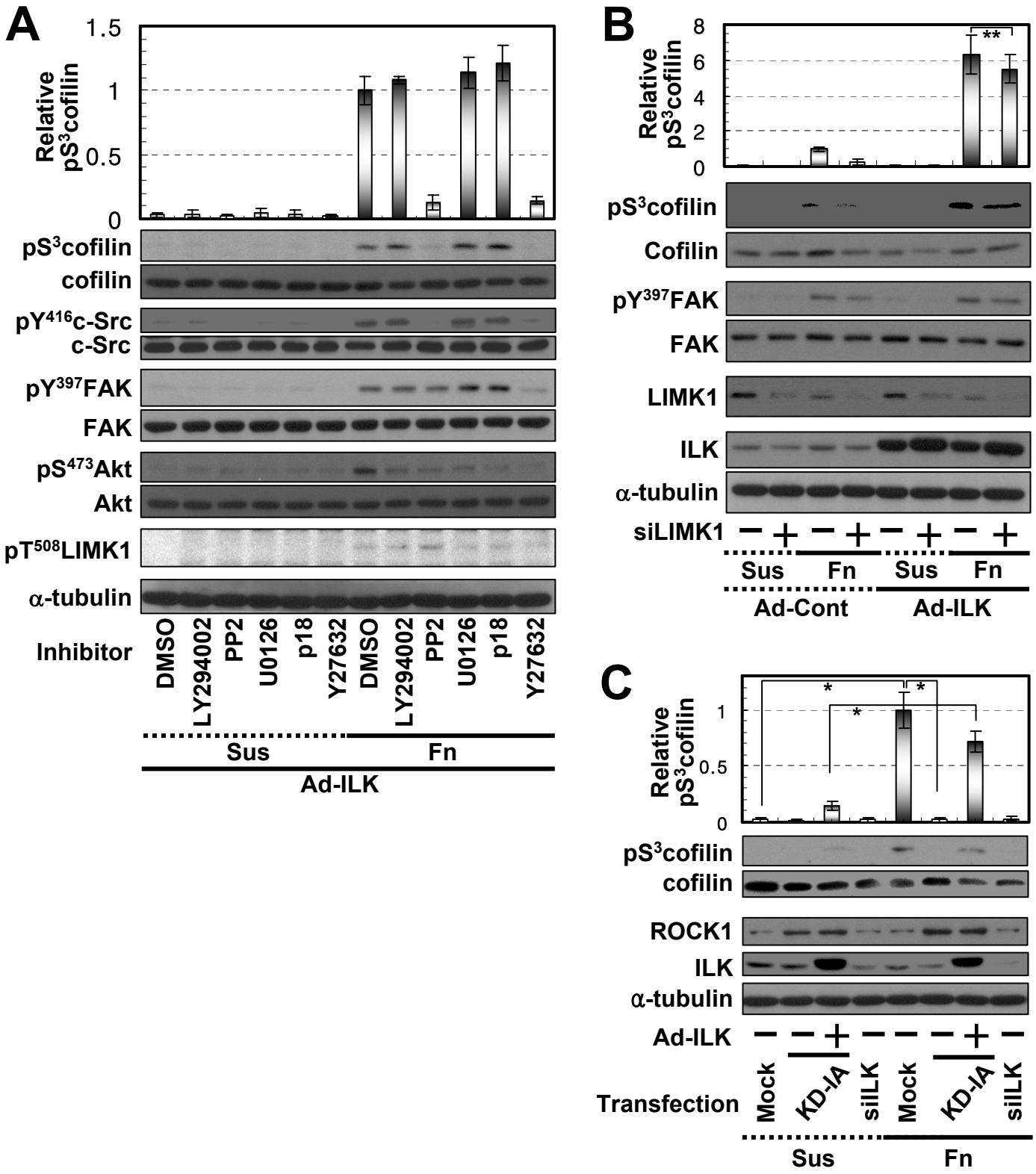

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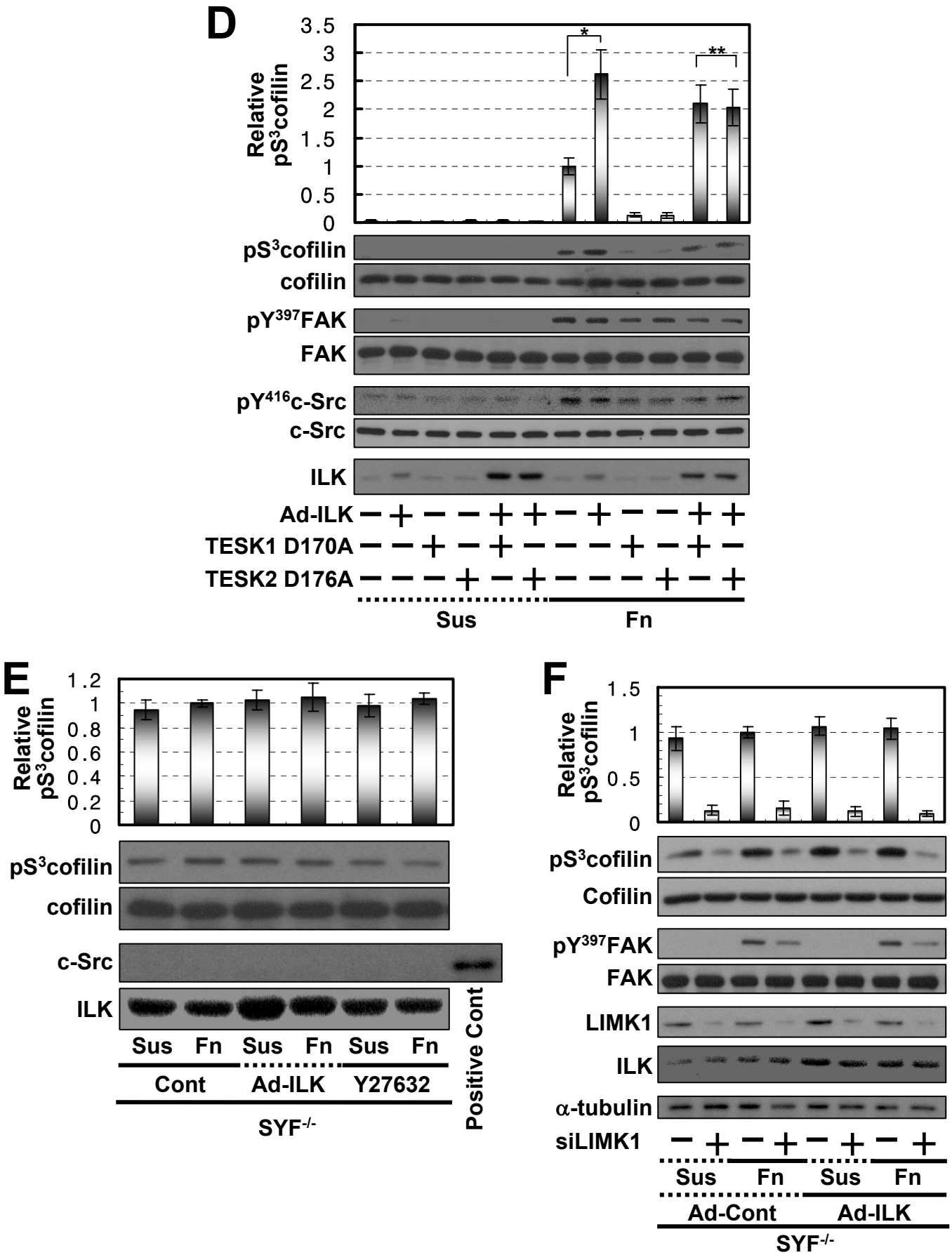

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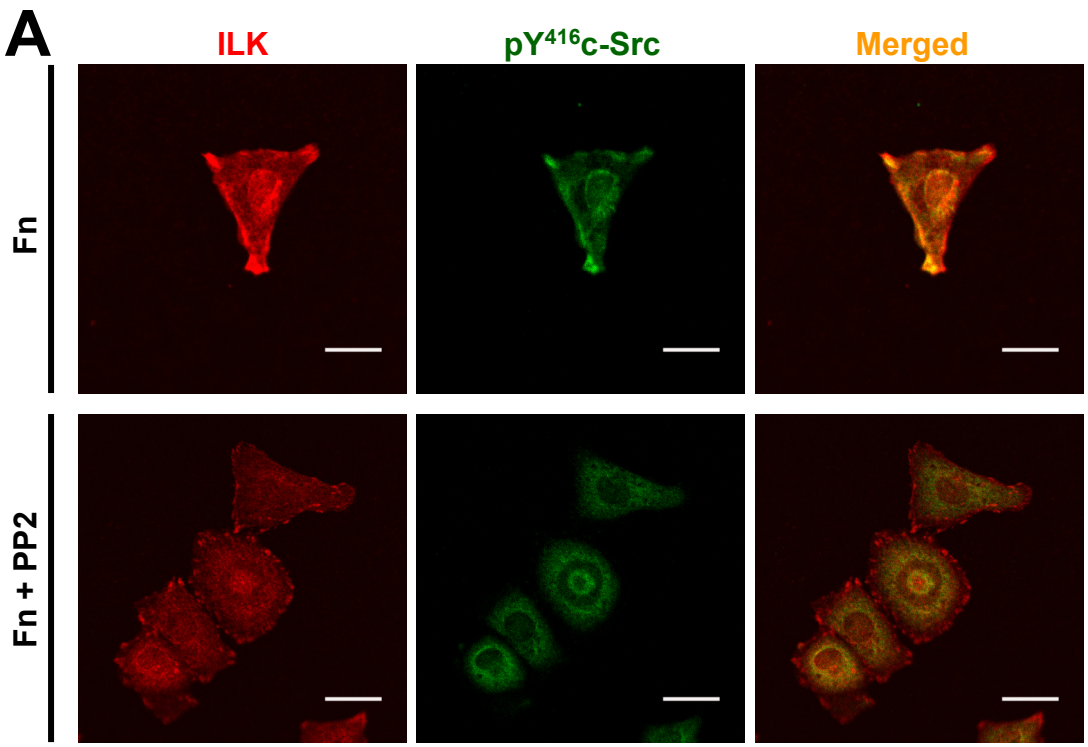

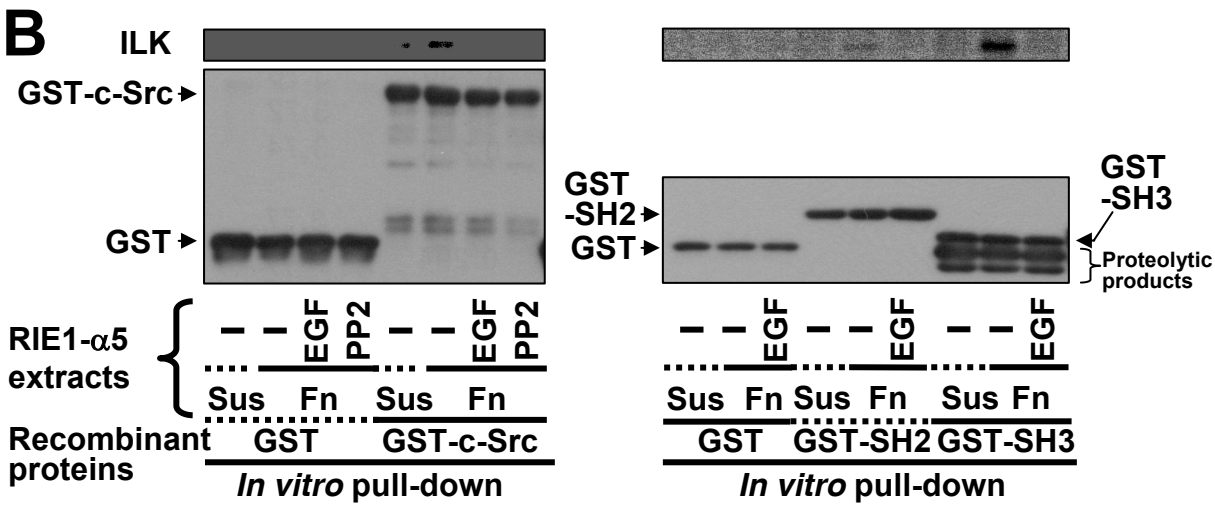

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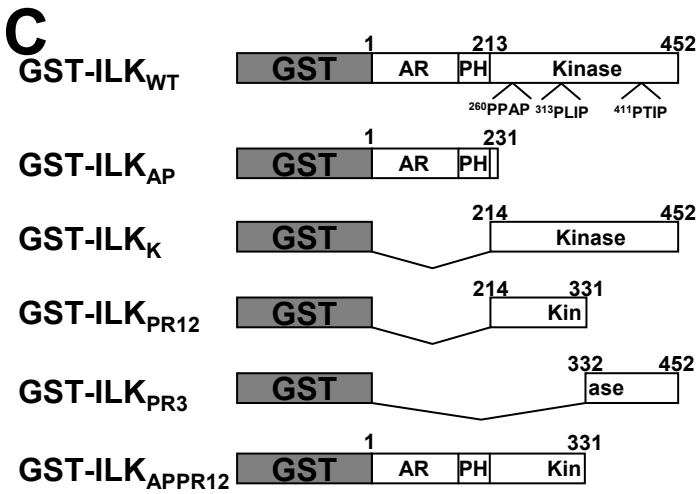

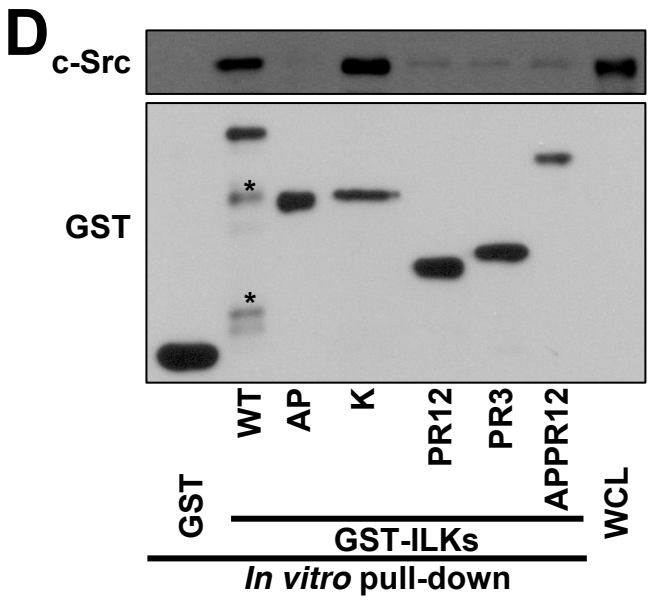

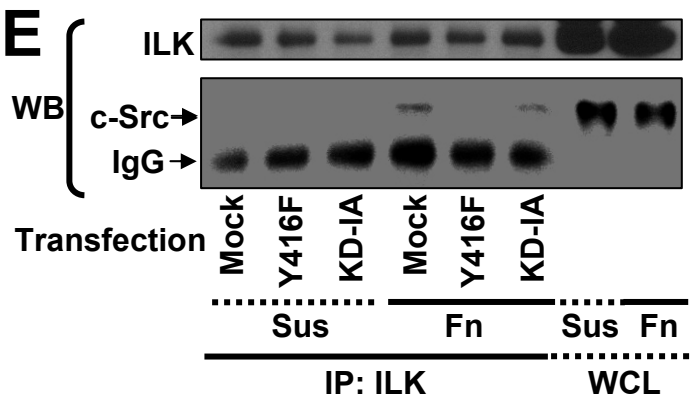

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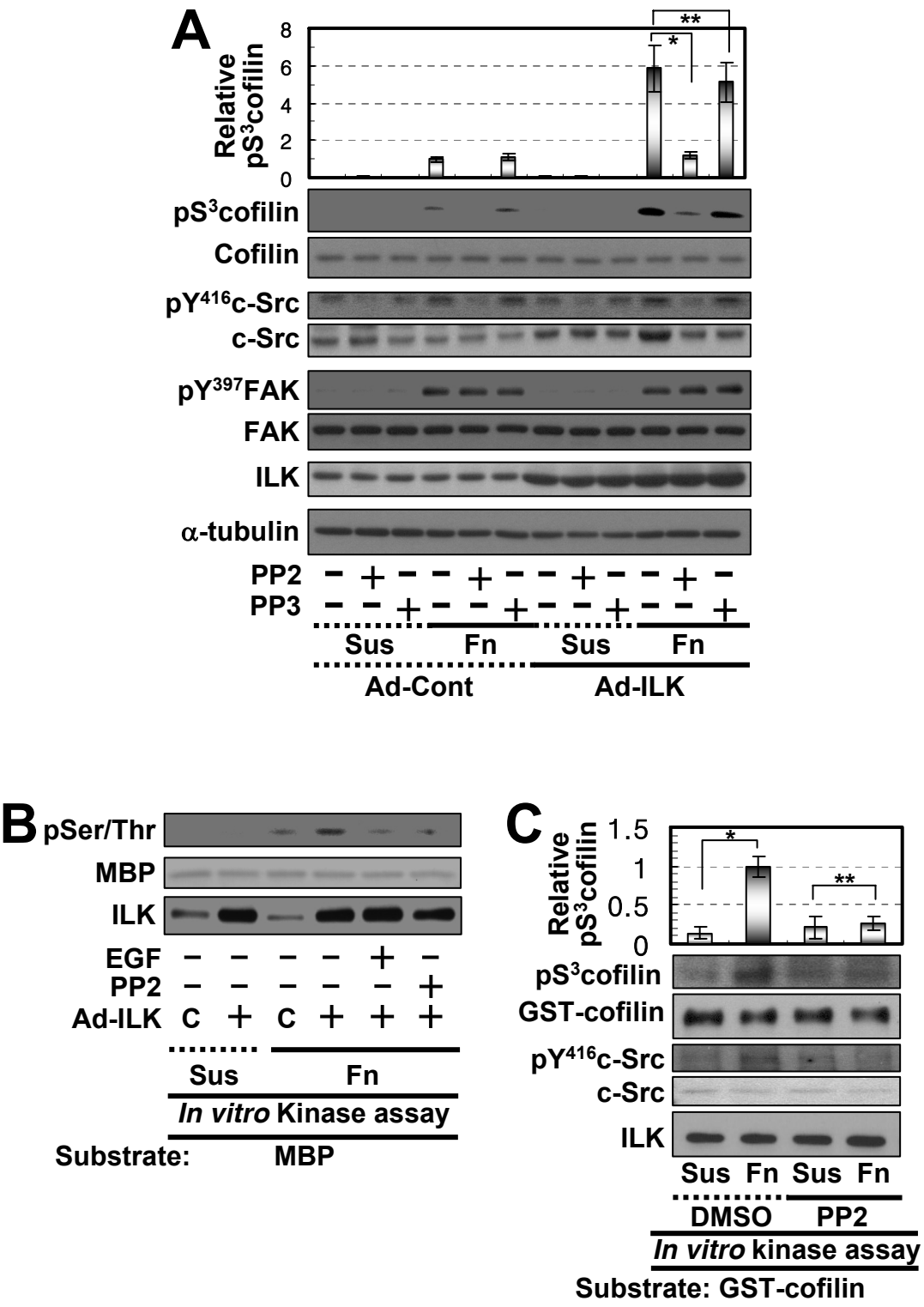

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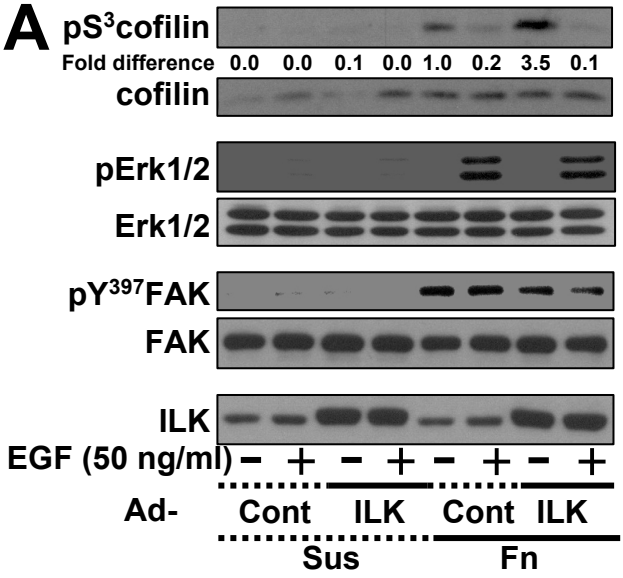

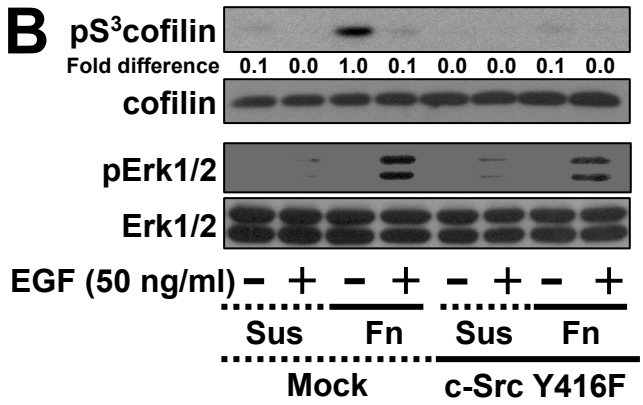

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