

Overexpression of Hyperactive Integrin-Linked Kinase Leads to Increased Cellular Radiosensitivity

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ABSTRACT

Integrin-linked kinase (ILK), bound to the cytoplasmic tails of integrin $\beta 1$, $\beta 2$, and $\beta 3$, is thought to signal through AKT and glycogen synthase kinase-3 β (GSK-3 β) for survival and proliferation regulation. To determine the role of ILK in the cellular radiation response, stably transfected A549 lung cancer cells overexpressing either wild-type (ILK-wk) or hyperactive ILK (ILK-hk) were studied for survival, signaling, proliferation, and examined in immunofluorescence and adhesion assays. Strong radiosensitization was observed in ILK-hk in contrast to ILK-wk mutants and empty vector controls. ILK small interfering RNA transfections showed radioresistance similar to irradiation on fibronectin. AKT, GSK-3 β -cyclin D1, mitogen-activated protein kinase 1/2-mitogen-activated protein kinase, and c-Jun NH₂-terminal kinase signaling was dysregulated in irradiated ILK-hk mutants. Immunofluorescence stainings of ILK-hk cells indicated disturbed ILK and paxillin membrane localization with concomitant decrease in focal adhesions. Profound ILK-hk-dependent changes in morphology were characterized by spindle-like cell shape, cell size reduction, increased cell protrusions, strong formation of membranous f-actin rings, and significantly reduced adhesion to matrix proteins. Additionally, ILK-wk and ILK-hk overexpression impaired $\beta 1$ -integrin clustering and protein Tyr-phosphorylation. Taken together, the data provide evidence that ILK signaling modulates the cellular radiation response involving diverse signaling pathways and through changes in f-actin-based processes such as focal adhesion formation, cell adhesion, and spreading. Identification of ILK and its signaling partners as potential targets for tumor radiosensitization might promote innovative anticancer strategies by providing insight into the mechanism of cell adhesion-mediated radioresistance, oncogenic transformation, and tumor growth and spread.

INTRODUCTION

Attachment of cells to extracellular matrix proteins is essential for proper execution and regulation of survival, proliferation, adhesion, migration, differentiation, cytoskeletal organization, and tissue organization (1–5). Recently, the important role of integrin-mediated cell-matrix interactions for prosurvival and antiapoptotic signaling in case of genotoxic injury by ionizing radiation and drugs has been strongly emphasized (6–12).

Integrins are a family of 18 α - and 8 β -subunit transmembrane cell surface adhesion receptors that connect the extracellular matrix and the actin cytoskeleton (13–15). Twenty-four known α/β -heterodimeric integrin receptors can be formed to fulfill different functions depending on cell type and cellular context. Adhesion of cells to a substratum induces the formation of multiprotein membrane complexes called focal adhesions. These complexes are composed of cell adhesion molecules such as integrins and growth factor receptors (14, 15). Colocalization and recruitment of adapter proteins and cytoplasmic protein kinases permit intensive and mutual cross-talk between these receptors and their signal transduction pathways for the regulation of many critical cell functions (1, 2, 16–20).

In vitro, the $\beta 1$ -integrin cytoplasmic domain binds a great variety of signaling and structural proteins such as integrin-linked kinase (ILK) and focal adhesion kinase as well as paxillin and α -actinin (21, 22). ILK is a serine/threonine kinase and interacts with the cytoplasmic tails of $\beta 1$ -, $\beta 2$ -, and $\beta 3$ -integrins (23). Although differentially expressed in normal tissues, ILK demonstrates constitutive overexpression in colorectal (24) and ovarian cancers (25) and melanoma (26), which is accompanied by constitutive phosphorylation of its downstream targets AKT and glycogen synthase kinase-3 β (GSK-3 β ; Ref. 27).

AKT is a serine/threonine kinase participating in cell survival, apoptosis, and proliferation signaling (28–30). Phosphorylation of both amino acid residues Thr³⁰⁸ and Ser⁴⁷³, through the phosphatidylinositol-3-kinase-dependent kinases PDK1 (31) and PDK2 (32), respectively, is required for full kinase activation. GSK-3 β is a negative regulator of Wnt and growth factor receptor signaling. Phosphorylation of GSK-3 β at Ser⁹ leads to inactivation (33). Interactions with β -catenin and subsequent association of β -catenin with Lef1/TCF transcription factors results in the induction of, for example, the *cyclin D1* gene (33, 34). The hypothesis that AKT and GSK-3 β are ILK targets provided an explanation of how integrin-mediated cell survival and cell proliferation signals could be transmitted.

That ILK is also involved in the regulation of focal adhesion formation, adhesion, spreading, and proliferation as well as subcellular aggregation and dynamics of actin stress fibers (35, 36) is based on its multiple interactions with other structural and signaling molecules such as paxillin (37), the double zinc finger domain (LIM)-only adaptor proteins PINCH-1 and PINCH-2 (38), and a new family of f-actin binding proteins termed parvins (39–41). Although genetic studies in *mouse*, *Drosophila*, and *Caenorhabditis elegans* confirmed these data, the exact kinase function of ILK remains elusive (36, 42, 43). For example, ILK-deficient flies demonstrated unchanged GSK-3 β and AKT phosphorylation, no patterning defects typical of impaired Wnt signaling, and no increases in apoptosis (42). Most interestingly, reconstitution of an *ilk* gene without kinase activity in *ilk*^{-/-} cells completely rescued the observed adhesion, spreading, and proliferation defects (35, 36).

The rationale for studying the role of ILK in radiosensitization is based on our previous findings that show cell adhesion-mediated radioresistance to be differentially influenced by substratum-dependent radiation-mediated activation of ILK, AKT, and GSK-3 β (8). Furthermore, the dependence of radiosensitization on ILK-mediated alterations of cell morphology is elucidated. For this study, A549 lung cancer cells were stably transfected with ILK-wild-type (ILK-wk)- or ILK-hyperactive kinase (ILK-hk)-overexpressing forms or empty vectors. We examined the effects of this manipulation on radiation survival, AKT-GSK-3 β , mitogen-activated protein kinase kinase 1/2-mitogen-activated protein kinase (MEK1/2-MAPK) and c-Jun NH₂-terminal kinase (JNK) signaling, ILK and paxillin subcellular localization, $\beta 1$ -integrin clustering, and clustering-induced protein Tyr-phosphorylation.

MATERIALS AND METHODS

Antibodies and Reagents. The following affinity purified mono- or polyclonal antibodies were purchased from commercial sources: anti-ILK (65.1.9),

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anti-PKB α /AKT, anti-phospho-tyrosine (4G10), antimouse IgG1 and IgG2a, and antirat IgG1 (Upstate, Hamburg, Germany); anti-phospho-PKB α /AKT-Ser⁴⁷³, anti-phospho-PKB α /AKT-Thr³⁰⁸, anti-phospho-GSK-3 β -Ser⁹, anti-phospho-MEK1/2, anti-MEK1/2, anti-phospho-MAPK, anti-MAPK, and anti-pRb-Ser⁷⁹⁵ (Cell Signaling, Frankfurt a.M., Germany); anti-pRb and anti-phospho-JNK (Santa Cruz Biotechnology, Heidelberg, Germany); anti- β -actin

and anti-paxillin (Sigma-Aldrich GmbH, Taufkirchen, Germany); Cy2-conjugated AffiniPure goat antimouse IgG and Cy3-conjugated AffiniPure goat antirat IgG (Dianova, Hamburg, Germany); anti- β 3-integrin (RUU-PL7F12) from Becton Dickinson (Heidelberg, Germany); and anti- β 1-integrin (K20) and rabbit antimouse IgG FITC from Dako (Hamburg, Germany). Anti- β 1-integrin antibodies (mAb13) were a generous gift of Dr. Kenneth M. Yamada

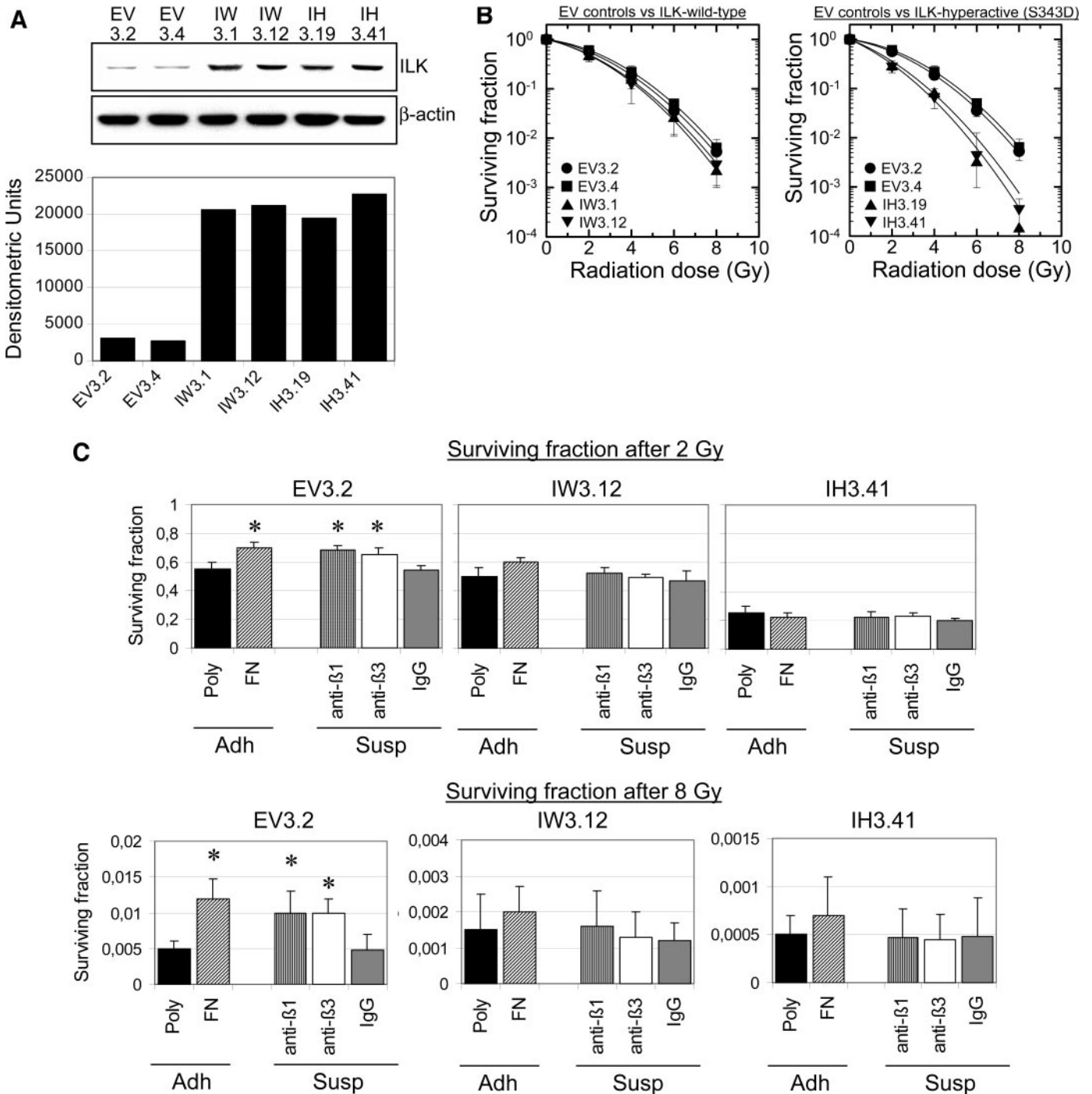


Fig. 1. A, Western blot analysis was performed on protein samples harvested at the time of the survival curves showing overexpression of ILK in the different cell clones compared with empty vector controls. β -Actin served as loading control. B, ILK-hk (S343D; IH3.19, IH3.41) mutants are significantly ($P < 0.01$) radiosensitized relative to empty vector controls (EV3.2, EV3.4) and ILK-wk mutants (IW3.1, IW3.12). The fit of the dose-effect curves was calculated using the linear quadratic model ($\log S = -\alpha D - \beta D^2$). Results are expressed as mean \pm SD of three independent experiments. C, ILK mutants are unsusceptible to radiosensitization modification by integrin ligand binding as compared with empty vector controls. Empty vector controls showed significant ($P < 0.01$) increase in radioresistance when adhered to fibronectin (FN) or stimulated by anti-integrin antibodies. Growth of empty vector controls, ILK-wk and ILK-hk mutants on FN versus polystyrene (Poly) as well as exposure of suspension cultures to anti- β 1-integrin (K20; 10 μ g/ml) or anti- β 3-integrin (RUU-PL7F12; 10 μ g/ml) antibodies versus unspecific IgGs, was examined. Columns represent mean \pm SD of three independent experiments. D, determination of surviving fractions of 2- or 8-Gy irradiated ILK siRNA-transfected EV3.2 controls revealed significant ($P < 0.01$) increase in radioresistance similar to FN and relative to nonspecific Duplex II (DII)- or untransfected cells (mock). Western blotting shows reduction of ILK expression after siRNA transfection. Columns represent mean \pm SD of three independent experiments. *, $P < 0.01$.

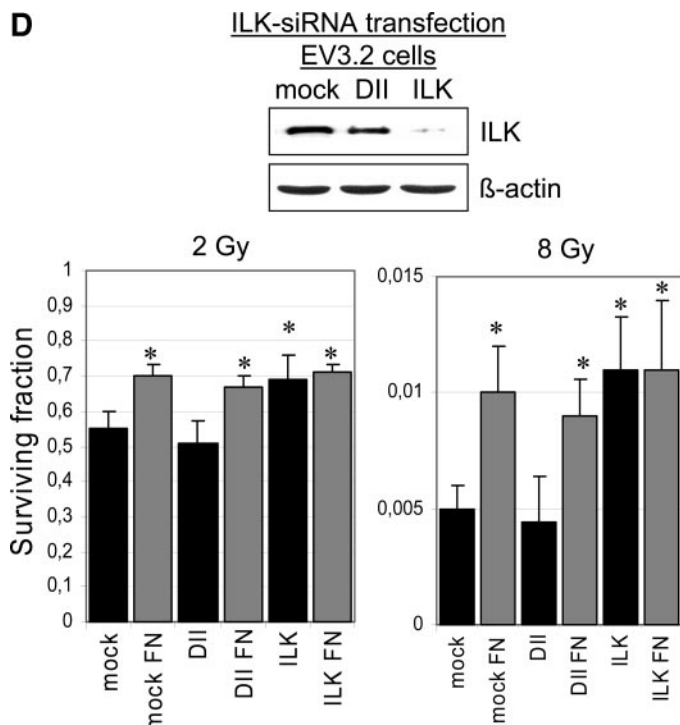


Fig. 1 Continued.

(National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD). FITC conjugated phalloidin for f-actin (Sigma-Aldrich GmbH). For protein detection, specific horseradish peroxidase-conjugated goat antirabbit and rabbit antimouse antibodies were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence reagent was from Amersham (Freiburg, Germany). β 1/ β 3-Integrin-blocking disintegrin Echistatin and G418 were purchased from Sigma-Aldrich GmbH, 4',6-diamidino-2-phenylindole from Serva (Heidelberg, Germany), and GRGDS (H-Gly-Arg-Gly-Asp-Ser-OH) and GRADSP (H-Gly-Arg-Ala-Asp-Ser-Pro-OH) peptides from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Fibronectin was from Becton Dickinson, laminin from Sigma-Aldrich GmbH, and vitronectin and collagen-III from Calbiochem-Novabiochem GmbH.

Stable Transfection and Cell Culture. Human A549 lung cancer cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were stably transfected using electroporation. pUSEamp vectors (Upstate) containing cDNA constructs encoding for ILK-hk or ILK-wk under the control of a cytomegalovirus promoter and the empty control vector (pUSE-amp+) were used. Hyperactive kinase activity was achieved by a double point mutation at Ser³⁴³ generated by a substitution of TC for GA at nucleotides 1027 and 1028 within the cDNA. Exponentially growing cells were prepared for transfection by washing and incubation on ice for 10 min in the presence of 1 μ g of plasmid/ 5×10^6 cells. Cells were then electroporated at 200 V and 950 μ F. After 10 min of incubation on ice, cells were resuspended in pre-warmed DMEM (Life Technologies, Inc., Karlsruhe, Germany) containing glutamax-I (L-alanyl-L-glutamine), sodium pyruvate, 4500 mg/ml glucose, and pyridoxine supplemented with 10% fetal bovine serum (PAA, Linz, Austria) and 1% nonessential amino acids (Life Technologies, Inc.). During 4–6 weeks, stably transfected clones were selected under 1.5 mg/ml G418, isolated, and monitored for ILK expression by Western blotting. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% nonessential amino acids at 37°C in a humidified atmosphere containing 10% CO₂. The pH of the medium was 7.4. Where indicated, serum starvation of cells was performed using DMEM supplemented with 1% nonessential amino acids without fetal bovine serum. For all experiments, asynchronous growing cell cultures were used.

Small Interfering RNA (siRNA) Transfection. The sequence of ILK siRNA was selected based on a method described previously (44). The target sequence that effectively mediates silencing of ILK expression is 5'-AAGGTTCGAGACTGGAGTACA-3' (sense sequences). The 21-nucleotide synthetic

siRNA duplex was prepared by MWG Biotech AG (Ebersberg, Germany) based on Dharmacon 2'-ACE technology. EV3.2 cells were transfected with the ILK siRNA or a 21-nucleotide irrelevant RNA Duplex II as a control using oligofectamine (Invitrogen, Karlsruhe, Germany). Depletion of ILK was confirmed by Western blotting.

Radiation Exposure. Irradiation was delivered at room temperature using single doses of 240-kV X-rays (Isovolt 320/10; Seifert, Ahrensburg, Germany) filtered with 3-mm Beryllium. The absorbed dose was measured using a Duplex dosimeter (PTW, Freiburg, Germany). The dose-rate was \sim 1 Gy/min at 13 mA. Applied doses ranged from 0 to 8 Gy.

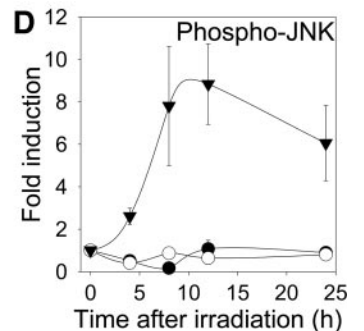
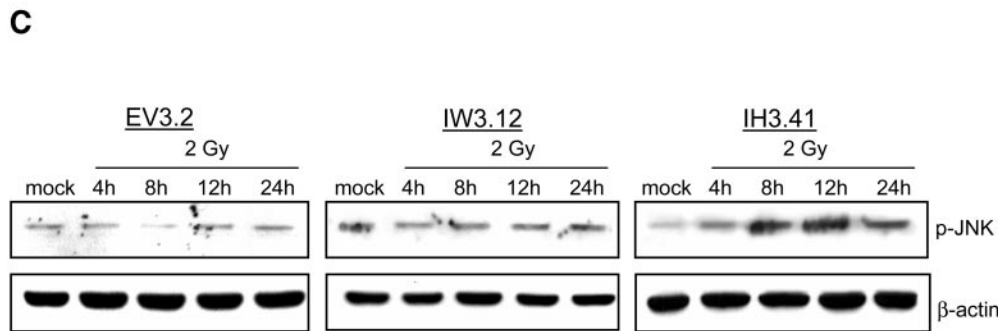
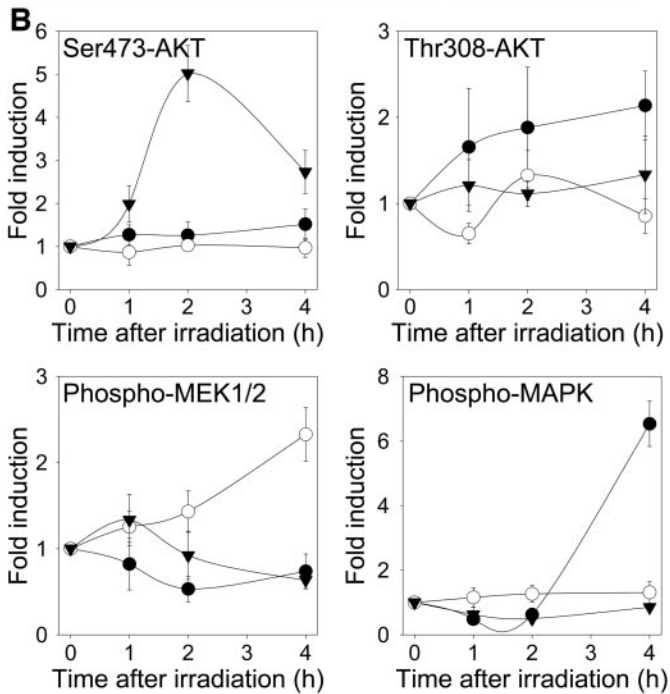
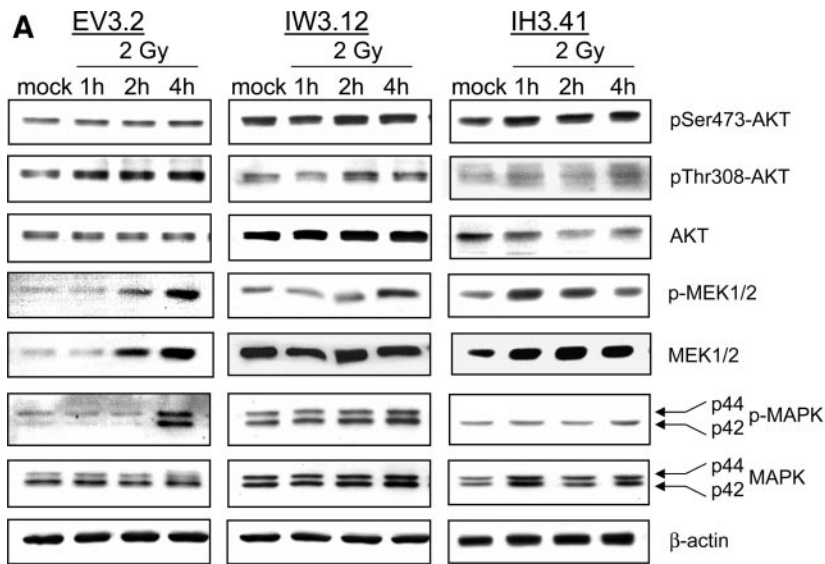
Colony Formation Assay. The colony formation assay was applied for measurement of clonogenic cell survival. Single cells were seeded in 6-well dishes. Immediately after cell attachment, cells were irradiated with 0–8 Gy. Where indicated, cells were plated onto fibronectin-coated polystyrene as described previously (45). For suspension cultures, cells were trypsinized and incubated with anti- β 1-integrin (K20; 10 μ g/ml) and anti- β 3-integrin (RUU-PL7F12; 10 μ g/ml) antibodies or unspecific IgGs (equivalent concentration) in serum-free medium for 1 h, irradiated and plated. After 8–10 days, colonies with >50 cells were stained with Coomassie blue and counted. All experiments were repeated three times ($n = 18$).

Total Protein Extractions and Western Blotting. At 1, 2, 4, 8, 12, and 24 h, nonirradiated and irradiated (2 Gy) cells were trypsinized, and cell lysis was performed using reducing modified radioimmunoprecipitation assay buffer. Radioimmunoprecipitation assay buffer contained 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA supplemented with protease inhibitor mixture complete (Roche, Mannheim, Germany), 5 mM sodium vanadate, and 5 mM sodium fluoride. Cell homogenization using a 25-gauge needle was followed by centrifugation. Amounts of total protein extracts were determined using BCA assay (Interchim, Montlucon Cedex, France) and stored at -134°C until use. Experiments were repeated three times. Ten percent SDS-PAGE gels were used to separate 20 μ g of total proteins extracts. Subsequent to transfer of proteins onto a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany), probing and detection of specific proteins were performed with described primary and secondary antibodies and enhanced chemiluminescence.

Immunofluorescence Staining and Laser Confocal Scanning Microscopy. A total of 10^3 cells was seeded onto fibronectin-coated Lab Tek chamber slides (Nalge Nunc International, Hamburg, Germany). Precoating was accomplished as published elsewhere (45). After a 24-h incubation of cells at 37°C in a humidified atmosphere containing 10% CO₂, cells were fixed using 2% paraformaldehyde in PBS and blocked with 2% goat serum. Staining of cells with anti-ILK (1:150) or anti-paxillin (1:150) antibodies or FITC-conjugated phalloidin for f-actin (1:300) was performed for 1 h at room temperature. Secondary antibodies, Cy2-conjugated AffiniPure goat antimouse IgG and Cy3-conjugated AffiniPure goat antirat IgG, were used at a dilution of 1:100. 4',6-Diamidino-2-phenylindole staining was accomplished at a dilution of 1:100. Fluorescence images were obtained using a Leica MRC1024 confocal laser scan microscope equipped with Leica Confocal Software (Leica Microsystems, Wetzlar, Germany).

β 1-Integrin Clustering and Protein Tyrosine Phosphorylation. Integrin clustering was examined according to a method described by Kornberg *et al.* (46). A total of 10^4 cells was seeded onto Lab Tek chamber slides. At 24 h after plating, cells were stimulated with specific K20 anti- β 1-integrin antibodies (1:100) for 30 min at 37°C-10% CO₂. Subsequently, secondary antibodies, Cy2-conjugated AffiniPure goat antimouse IgG (1:100), were used to visualize β 1-integrin clusters. This step was performed on ice for 30 min. Fixation of cells was performed and fluorescence images were obtained as described above. Analysis of protein Tyr-phosphorylation was performed on replicate cultures. After antibody stimulation, cells were harvested, lysed, and prepared for total Tyr-phosphorylation analysis by Western blotting. Control cluster and Tyr-phosphorylation experiments were performed using unspecific IgG1 antibodies.

Adhesion Assay. Cell adhesion to fibronectin, laminin, vitronectin, and collagen-III was studied according to a method described previously (47). In brief, 5×10^4 cells were plated on fibronectin-, laminin-, vitronectin-, and collagen-III-precoated 96-well plates (Nalge Nunc International) in serum-free medium for 1 h. Additional experiments were accomplished in the presence of adhesion-blocking anti- β 1-integrin mAb13 antibodies (1 μ g/ml), β 1/ β 3-inte-



grin-blocking disintegrin Echistatin (10 μ M; Ref. 48), and GRGDS peptides (500 μ g/ml). Controls were exposed to equivalent concentrations of unspecific antirat IgG1 antibodies or inactive control peptide GRADSP. After 1 h, nonadherent cells were withdrawn by gentle washing with PBS. Adherent cells were fixed with 70% ethanol and stained with 1% methylene blue. Washing

with distilled water was followed by the addition of 100 μ l of a 0.1 M HCl solution to each well and measurement of absorbance of the resulting solution at 630 nm using a spectral-photometer (Spectra max 190; Molecular Devices, Ismaningen, Germany). All experiments were performed in triplicate and repeated three times.

Data Analysis. Means \pm SD of surviving fractions, induction of protein phosphorylation, and cell adhesion were calculated with reference to untreated controls defined as 1.0 or in a percentage scale. To test statistical significance, ANOVA was performed with a software package (Microsoft Excel 97) on IBM computer systems. Results were considered statistically significant if P of <0.05 was reached. The fit of the dose-effect curves was calculated using the linear quadratic model ($\log S = -\alpha D - \beta D^2$).

RESULTS

Overexpression of Hyperactive ILK Leads to Radiosensitization. To determine the cellular radiosensitivity of ILK-wk- and ILK-hk-overexpressing mutant and empty vector controls, clonogenic survival of both adhesion and suspension cultures was measured after irradiation. Additionally, clonogenic survival was measured after plating of cells on fibronectin and after ILK-siRNA transfection of empty vector controls.

In Fig. 1A, expression levels of endogenous ILK in empty vector controls [EV3.2, EV3.4; similar to parental A549 cells (8)] and overexpressed ILK-wk (IW3.1, IW3.12) and ILK-hk (IH3.19, IH3.41) in selected clones were examined by Western blotting. ILK overexpression was ~ 7 -fold in ILK clones as compared with empty vector cells.

Overexpression of ILK-hk significantly ($P < 0.01$) radiosensitized IH3.19 and IH3.41 cells at radiation doses ≥ 2 Gy relative to empty vector controls (EV3.2, EV3.4) and ILK-wk mutants (IW3.1, IW3.12; Fig. 1B). The cellular radiosensitivity of empty vector controls was similar to the parental A549 cell line (8).

Incubation of EV3.2 suspension cultures with stimulating anti-integrin antibodies resulted in significantly ($P < 0.01$) increased radiation survival rates at 2 and 8 Gy similar to survival rates on fibronectin (Fig. 1C). In contrast, this fibronectin- or antibody-related improvement of radiation survival (2 or 8 Gy) was not detectable in ILK-wk and ILK-hk mutants (Fig. 1C). Nonspecific IgGs had no effect on survival as compared with polystyrene cultures.

Similar to fibronectin, silencing of ILK expression in EV3.2 cells by siRNA transfection resulted in significantly ($P < 0.01$) increased survival after 2 or 8 Gy relative to survival of nonspecific Duplex II or untransfected cells (Fig. 1D). Additionally, irradiation of ILK siRNA- or Duplex II-transfected cells on fibronectin showed significantly ($P < 0.01$) elevated survival rates (Fig. 1D). Duplex II RNA treatment did not significantly alter the plating efficiencies of tested cells.

AKT, MEK1/2, MAPK, and JNK Expression and Phosphorylation Are Modulated after Irradiation in an ILK-Dependent Manner. ILK is thought to phosphorylate AKT on Ser⁴⁷³. Although AKT and JNK are important regulators of survival, MEK1/2 and MAPK mainly serve in regulation of proliferation.

AKT expression was not affected in irradiated EV3.2 and IW3.12 cells (Fig. 2A). IH3.41 cells showed a strong transient radiation-dependent reduction of AKT at 2 h (Fig. 2A). Basal phospho-Ser⁴⁷³-AKT was increased in both IW3.12 and IH3.41 cells as compared with EV3.2 cells (Fig. 2A). Interestingly, phospho-AKT-Ser⁴⁷³ showed only a minor radiation-dependent increase, but AKT protein was greatly reduced. This resulted in a relative 5-fold induction of phospho-AKT-Ser⁴⁷³ in ILK-hk mutants (Fig. 2, A and B). Basal

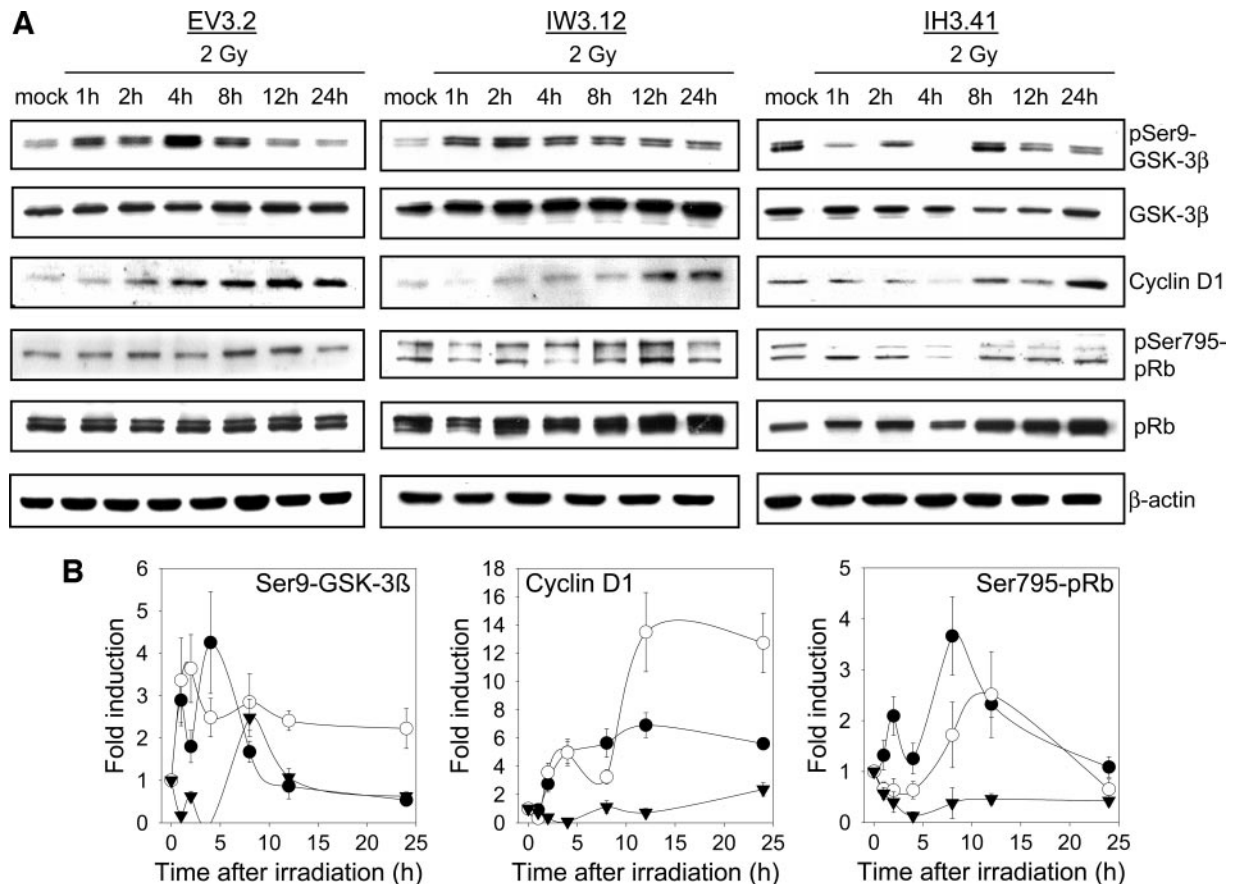


Fig. 3. A, nonirradiated (mock) and 2-Gy irradiated ILK mutants showed altered expression and phosphorylation of GSK-3 β , cyclin D1, and pRb at indicated time points. ILK-hk-overexpressing IH3.41 cells showed basal elevation of phospho-GSK-3 β -Ser⁹ and cyclin D1. Cyclin D1 expression and pRb phosphorylation could be correlated with radiation-altered GSK-3 β phosphorylation. β -Actin served as loading control. B, densitometry of Ser⁹-GSK-3 β , cyclin D1, and Ser⁷⁹⁵-pRb (triplet in IW3.12 cells; doublet in IH3.41 cells) bands was performed, and fold inductions (mean \pm SD) were calculated in relation to unirradiated controls after normalization to β -actin. ●, EV3.2; ○, IW3.12; ▼, IH3.41.

phospho-Thr³⁰⁸-AKT was similar in EV3.2 and IW3.12 cells but reduced in IH3.41 cells, which is concluded from delayed chemiluminescence-mediated visualization. After radiation, phospho-Thr³⁰⁸-AKT was induced by 1.7-fold at 1 h in EV3.2 cells but remained unchanged in IW3.12 and IH3.41 cells (Fig. 2, A and B).

Changes in MEK1/2 phosphorylation directly corresponded to changes in total MEK1/2 expression in irradiated empty vector controls and ILK-hk mutants (Fig. 2, A and B). In ILK-wk mutants, constant MEK1/2 expression was accompanied by a radiation-dependent 2.3-fold induction of phospho-MEK1/2, which indicates an increase in MEK1/2 activity (Fig. 2, A and B).

MAPK expression remained unaffected in EV3.2 cells but was induced in IW3.12 and IH3.41 cells at 4 or 1 h after irradiation, respectively (Fig. 2, A and B). In contrast to radiation-induced phospho-p42/p44-MAPK (6.5-fold) in EV3.2 cells, MAPK phosphorylation remained constant in irradiated IW3.12 cells. Most interestingly,

unirradiated and irradiated ILK-hk-overexpressing IH3.41 cells showed a lack of phospho-p44-MAPK as well as unaltered phospho-p42 MAPK levels (Fig. 2, A and B).

As shown in Fig. 2, C and D, phosphorylation of JNK was greatly induced by 8-fold in irradiated ILK-hk mutants relative to irradiated empty vector controls and ILK-wk cells. Interestingly, phospho-JNK was almost completely abrogated at 8 h in 2-Gy irradiated EV3.2 cells.

ILK Overexpression Differentially Modulate GSK-3 β , Cyclin D1, and Retinoblastoma Protein (pRb) after Irradiation. The phosphorylation status of the ILK downstream target GSK-3 β affects cyclin D1 proteolysis, and in conjugation with pRb, these proteins are essential for the regulation of G₁-phase transition (3).

After irradiation, GSK-3 β remained stable in EV3.2 cells, was induced by 1.4-fold in IW3.12, and reduced by 2-fold in IH3.41 cells (Fig. 3A). Because of hyperactive ILK, basal GSK-3 β -Ser⁹ phospho-

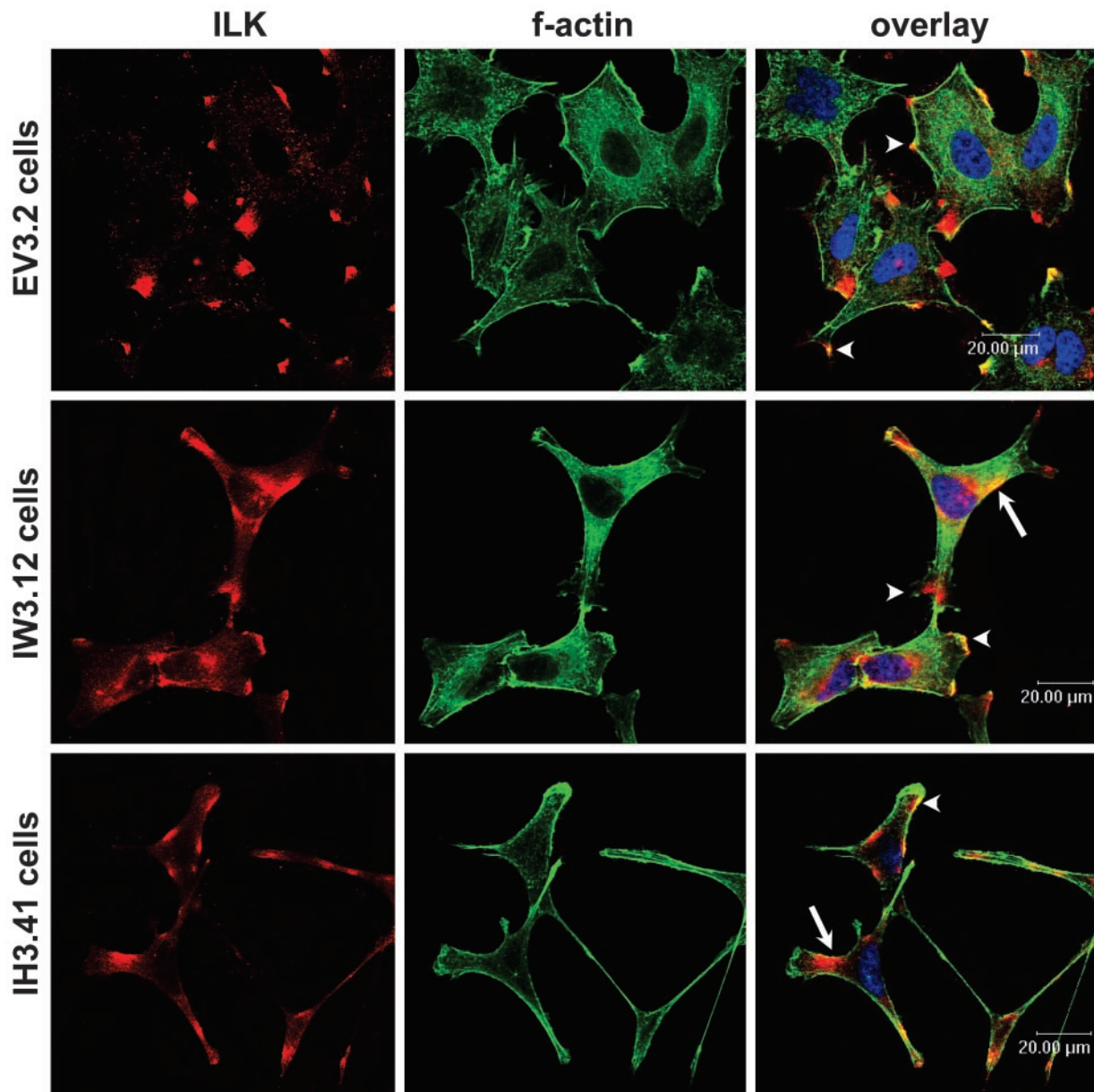


Fig. 4. ILK overexpression of wild-type (IW3.12) or hyperactive kinase (IH3.41) forms induced accumulation of cytoplasmic perinuclearly located ILK as compared with empty vector controls (EV3.2). Decrease of membranous ILK was accompanied by decreased ILK-f-actin colocalization. Immunofluorescence stainings were visualized by confocal scanning microscopy after fixing and staining of cells with the appropriate antibodies and FITC-conjugated phalloidin for f-actin. Additionally, ILK-f-actin stainings were combined with 4',6-diamidino-2-phenylindole staining of nuclei. *Arrowheads* denote membrane areas of protein colocalization; *arrows* denote cytoplasmic localization. *Bar*, 20 μ m.

rylation was elevated in IH3.41 cells as compared with empty vector controls and IW3.12 cells (Fig. 3A). Although radiation strongly induced Ser⁹ phosphorylation of GSK-3 β in EV3.2 and IW3.12 cells within 1 h, irradiated IH3.41 cells showed a reversion to basal levels at 12 h after a complete loss at 4 h and a 2.4-fold induction at 8 h (Fig. 3, A and B).

In parallel to radiation-stimulated GSK-3 β phosphorylation of EV3.2 and IW3.12 cells, cyclin D1 was induced by >2-fold within 1 h (Fig. 3, A and B). IW3.12 mutants showed pronounced cyclin D1 induction by >13-fold until 12 h after irradiation (Fig. 3, A and B). Contrary to ILK-wk mutants, basal cyclin D1 expression was elevated in ILK-hk cells, and irradiation led to a complete abrogation of cyclin D1 at 4 h before reinduction at 8 h (Fig. 3, A and B).

Interestingly, pRb migrated as a doublet in EV3.2 and as a triplet in IW3.12 but not in IH3.41 cells (Fig. 3A). Although EV3.2 cells lacked radiation-dependent pRb modification and IW3.12 cells displayed only slight modification, IH3.41 cells showed strong induction of pRb after irradiation (Fig. 3A). Phosphorylation of pRb at Ser⁷⁹⁵ was induced in irradiated EV3.2 and IW3.12 cells but strongly reduced in IH3.41 mutants (Fig. 3, A and B). In EV3.2 cells, the phospho-pRb signal corresponded to the lower total pRb protein band. In IW3.12 cells, the two phospho-pRb signals corresponded to the upper and lower pRb protein bands of a pRb triplet (Fig. 3, A and B). In IH3.41 cells, the lower phospho-pRb band matched with the single total pRb signal. Identification of the higher phospho-pRb signal detected in the ILK-hk mutants failed using anti-pRb antibodies (Fig. 3A).

ILK Overexpression Induces Changes in Subcellular Localization of ILK Itself, Paxillin, and F-Actin, Formation of Focal Adhesions, and Cell Morphology. To examine ILK, paxillin and f-actin subcellular localization in cytoplasm, cell membrane and focal adhesions, confocal scanning microscopy was performed in cells grown on fibronectin.

ILK was predominantly located in focal adhesions of EV3.2 controls (Fig. 4, arrowheads). In IW3.12 and IH3.41 cells, ILK showed a pronounced cytoplasmic localization with a perinuclear maximum (Fig. 4, arrows). In IW3.12 cells, ILK also localized to a decreased number of focal adhesions and lamellipodia as compared with EV3.2 cells. In IH3.41 mutants, ILK membrane localization and focal adhesion number was further reduced (Fig. 4, arrowheads and arrows). Paxillin mainly localized to focal adhesions in EV3.2 cells and was barely detectable in the cytoplasm (Fig. 5, arrowheads). In contrast, paxillin was increasingly cytoplasmic in both ILK mutants. This was

accompanied by a decrease of membranous paxillin (Fig. 5, arrows). β 1-Integrin membrane and cytoplasmic localization was not disturbed in tested cell lines (data not shown). F-Actin staining showed a spread cell morphology and normal distribution of long and short actin fibers in EV3.2 and IW3.12 cells (Fig. 4). In IH3.41 cells, however, this pattern was replaced by a thick f-actin ring at the cytoplasmic face of the membrane and a pronounced decrease of short and long actin fibers (Fig. 4). Costaining of ILK or paxillin (data not shown) with f-actin confirmed normal formation of focal adhesions as well as colocalization of these proteins in EV3.2 controls and, although focal adhesion formation was reduced, in IW3.12 cells (Fig. 4). In contrast, ILK-hk-overexpressing IH3.41 cells did not exhibit ILK colocalization with f-actin (Fig. 4). Additionally, profound morphological changes were detected in ILK-hk cells characterized by reduction in cell size, fibroblastoid cell shape and increased undirected lamellipodia and filopodia (Figs. 4 and 5).

4',6-Diamidino-2-phenylindole staining was performed for detailed distinction between cytoplasmic and nuclear localization of ILK (Fig. 4). In control experiments using secondary antibodies, no fluorescence signals were detected (data not shown).

ILK Overexpressing Mutants Display Impaired β 1-Integrin Membrane Clustering and Clustering-Mediated Protein Tyrosine Phosphorylation. Stimulation of β 1-integrins with specific activating anti- β 1-integrin antibodies initiates the formation of β 1-integrin clusters in the cell membrane. This event leads to increased levels of protein tyrosine phosphorylation (46).

Although in anti- β 1-integrin antibody-stimulated EV3.2 cells β 1-integrins were strongly clustered at defined membrane sites, β 1-integrins remained diffusively distributed in the membrane of IW3.12 and IH3.41 cells (Fig. 6A, arrows). In contrast to EV3.2 cells, which demonstrated increased protein Tyr-phosphorylation after anti- β 1-integrin clustering, protein Tyr-phosphorylation was significantly ($P < 0.01$) reduced down to 65 and 51% in IW3.12 and IH3.41 cells, respectively (Fig. 6, B and C). Nonspecific IgG control experiments did not affect β 1-integrin clustering (data not shown) and protein Tyr-phosphorylation in tested cell lines (Fig. 6B).

ILK Hyperactive Kinase Mutants Show Reduced Adhesion to Matrix Proteins. Adhesion experiments were performed to investigate the impact of ILK on cell adhesion to various extracellular matrix proteins. These experiments focused on β 1-integrin-mediated binding.

Adhesion of EV3.2 and IW3.12 cells to fibronectin, laminin,

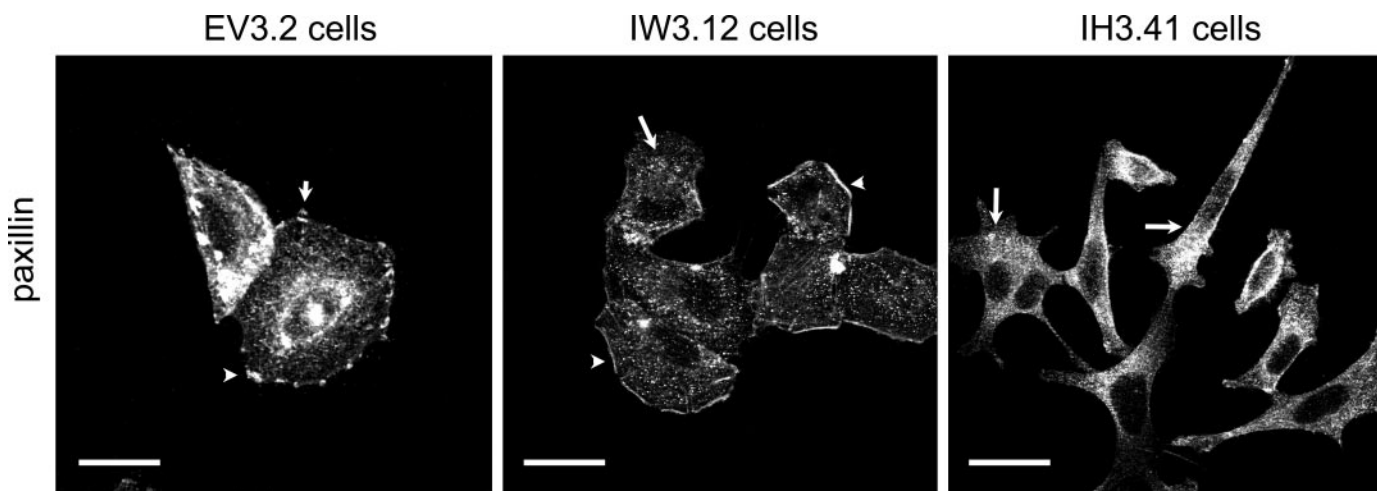


Fig. 5. Subcellular localization of paxillin is altered by ILK-wk (IW3.12) and, in particular, ILK-hk (IH3.41) forms. Although ILK-wk reduces paxillin content in focal adhesions and increases cytoplasmic paxillin levels, ILK-hk completely diminishes membranous paxillin and strongly elevates cytoplasmic localization. Arrowheads denote membrane areas of protein colocalization; arrows denote cytoplasmic localization. Bar, 20 μ m.

vitronectin, and collagen-III showed similar results (Fig. 7). Incubation of cells with anti- β 1-integrin antibodies (mAb13), Echistatin or GRGDS peptides significantly ($P < 0.01$) reduced adhesion to fibronectin by 5–7-fold in both cell lines (Fig. 7). Although blocking agents resulted in proportionally similar reduction of adhesion to fibronectin in IH3.41 cells, IH3.41 cells demonstrated a significant ($P < 0.01$) decrease in adhesion to fibronectin, vitronectin, and collagen-III by 1.7–2.3-fold in comparison with EV3.2 and IW3.12 cells (Fig. 7). Control IgG1 antibodies or unspecific GRADSP peptides did not influence adhesion to fibronectin of examined cell lines.

DISCUSSION

Cell adhesion to matrix proteins improves radiation cell survival. Integrins and downstream signaling via ILK are thought to be involved in this effect, but the exact molecular mechanisms are cur-

rently unclear. The major findings of this study are as follows: (a) strong radiosensitization of ILK-hk-overexpressing mutants relative to ILK-wk mutants and empty vector controls; (b) radioresistance through ILK siRNA transfection similar to radioresistance seen on fibronectin; (c) pronounced ILK-hk-dependent changes in AKT, MEK1/2-MAPK, JNK, and GSK-3 β -cyclin D1 signaling after irradiation; (d) disturbed ILK and paxillin membrane localization concomitant with reduction of focal adhesions in ILK-hk cells; (e) profound ILK-hk-dependent induction of cell size reduction and increased number of cell protrusions, which were accompanied by strong formation of membranous f-actin rings and significantly reduced adhesion to matrix proteins; and (f) ILK-dependent impairment of β 1-integrin clustering and protein Tyr-phosphorylation.

Whereas normal cells require adhesion for survival and growth, transformed cells often express an anchorage-independent growth

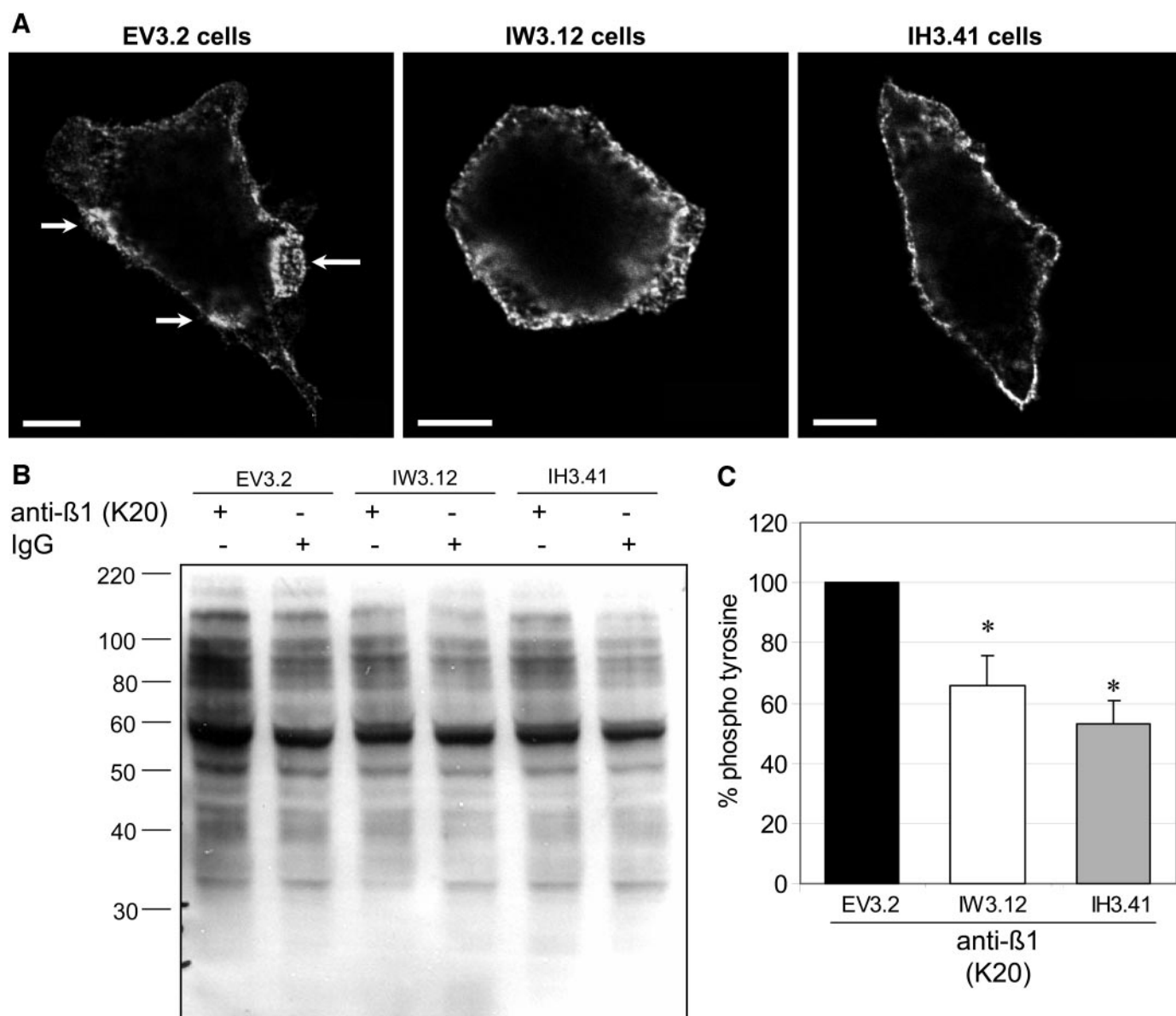


Fig. 6. A, antibody-induced β 1-integrin clustering is inhibited by ILK-wk and -hk overexpression. Cells were exposed to stimulating K20 anti- β 1-integrin antibodies to induce β 1-integrin clustering. β 1-Integrin localization was visualized by Cy3 secondary antibodies. Arrows denote β 1-integrin clusters in EV3.2 in contrast to IW3.12 and IH3.41 cells. Bar, 8 μ m. B, β 1-integrin clustering-mediated protein Tyr-phosphorylation is impaired ILK dependently. Total protein extracts of K20 anti- β 1-integrin antibody- or nonspecific IgG-stimulated cells were electrophorized and probed with anti-phospho-tyrosine antibodies. The experimental set-up was identical to the one used for immunofluorescence stainings. C, levels of protein Tyr-phosphorylation were measured densitometrically and revealed a significant ($P < 0.01$) reduction in IW3.12 and IH3.41 cells relative to EV3.2 controls. Columns indicate mean \pm SD of protein Tyr-phosphorylation of IW3.12 or IH3.41 cells relative to EV3.2 controls (= 100%). *, $P < 0.01$.

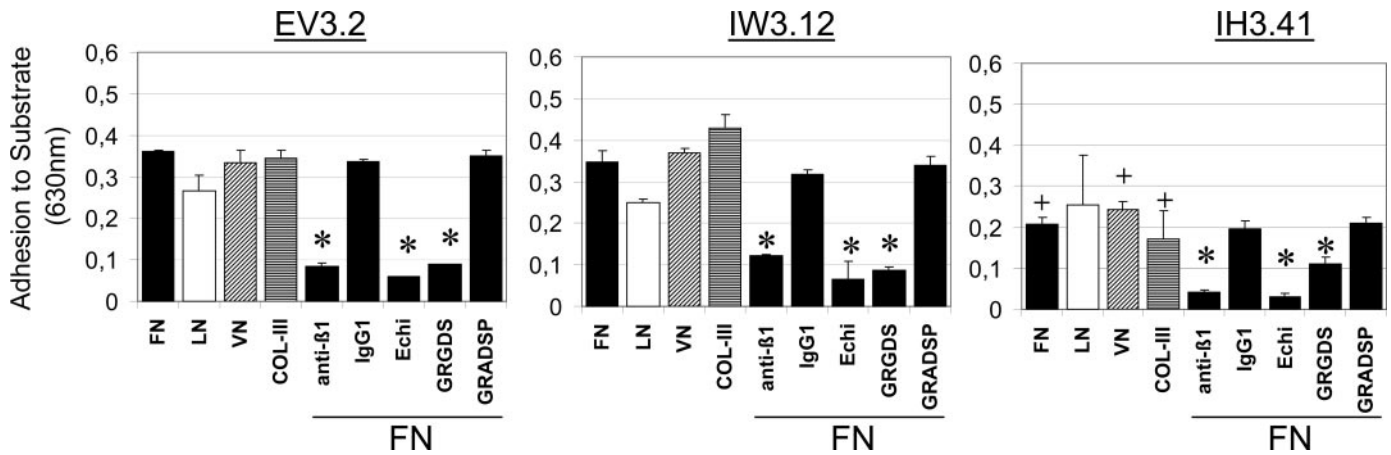


Fig. 7. Cell adhesion to extracellular matrix proteins is impeded in ILK-hk mutants. To determine adhesion capability and functional $\beta 1$ -integrin engagement, cells were seeded onto fibronectin (FN)-, laminin (LN)-, vitronectin (VN)-, and collagen-III (COL-III)-precoated polystyrene. For examination of cell adhesion to FN in more detail, specific blocking anti- $\beta 1$ -integrin antibodies (mAb13; 1 $\mu\text{g}/\text{ml}$), $\beta 1/\beta 3$ -integrin-blocking disintegrin Echinatin (Echi; 10 μM) or GRGDS peptides (500 $\mu\text{g}/\text{ml}$), were used. Control experiments were performed with equivalent concentrations of nonspecific antirat IgG1 antibodies or inactive control peptide GRADSP. Columns represent mean \pm SD of the absorbances at 630 nm representing cell adhesion to the different substrates. Statistical analysis compared, at first, adhesion to FN of nonblocked *versus* blocked cells (*, $P < 0.01$) and, secondary, adhesion of IH3.41 cells *versus* adhesion of empty vector controls (+, $P < 0.01$).

phenotype because of mutations in anchorage-related genes such as the small GTPase Rho or the Rho family members Cdc42 or Rac (49, 50). However, as shown by several groups (51–55), integrin-mediated cell-matrix interactions advantageously impact on normal and tumor cell survival after irradiation. This phenomenon is called cell adhesion-mediated radioresistance (9). On the basis of our reported observations that showed substratum-dependent, radiation-induced modification of the ILK-AKT-GSK-3 β signaling axis (8), an increased cellular radioresistance similar to fibronectin or other matrix proteins, was hypothesized by ILK overexpression. Most interestingly, overexpression of constitutively active ILK-optimized cell death pathways in terms of increased cellular radiosensitivity. These data are in strong agreement with recent findings in a variety of transformed cell lines that demonstrated the dependence of apoptosis-related cell death programs on cell adhesion (11, 12).

Signaling dysregulations observed in constitutively active ILK mutants with special concern to phospho-GSK-3 β and its downstream target cyclin D1, lack of p44-MAPK phosphorylation, strong phosphorylation of JNK, and alterations of phospho-pRb give evidence for an important role of ILK in the radiation response. Disproportional phosphorylation of Ser⁴⁷³ and Thr³⁰⁸ of AKT impede full kinase activation and downstream signaling to cell death inhibitory molecules such as Bad and caspase-9 (28). Similarly, loss of phospho-p44-MAPK is likely to disturb proliferation. Despite the fact that no radiation-induced apoptosis is detectable in parental A549 cells (8) and in ILK-transfected mutants, the tremendous increase in JNK phosphorylation is likely to participate in radiosensitization of ILK-hk cells. ILK's role in radiation survival was furthermore supported by ILK siRNA transfection, which resulted in radioresistance similar to fibronectin. In addition to p53, Arf, and c-Abl as important regulatory molecules in integrin signaling after genotoxic stress (11, 12), our findings uncovered ILK as negative regulator of integrin-mediated survival signaling.

That ILK in its constitutive active form negatively influences signaling for proliferation after irradiation was underscored by promoted dephosphorylation of GSK-3 β . This was paralleled by cyclin D1 and phospho-pRb down-regulation, events that led to a strong accumulation of G₁-phase cells.¹ GSK-3 β is a multifaceted protein kinase, and the prominent effects observed in this study suggest it as another critical molecule in the cellular response to genotoxic stress.

Additional studies will address this issue in the context of concerted integrin-growth factor receptor signaling.

ILK interacts not only with integrins and different signaling kinases but also with various structural proteins such as paxillin (5). Immunofluorescence stainings revealed that ILK-hk overexpression results in highly diminished membrane localization of focal adhesion-associated ILK and paxillin. The combination of a defective cytoskeletal actin organization and a reduction in focal adhesion formation accounted for the observed adhesion defects observed in ILK-hk mutants. Although the f-actin stainings also suggest an ILK-hk-related spreading defect, ILK mutants, contrary to published data (23), were not able to grow anchorage-independently.¹ These findings strongly underscore the hypothesis of an interdependence of adhesion and spreading with the cellular resistance against genotoxic agents.

As a result of impaired antibody-stimulated $\beta 1$ -integrin clustering in ILK mutants, cellular proteins were less Tyr-phosphorylated relative to empty vector controls. Because Tyr-phosphorylation of proteins is thought to represent signaling events, a reduced phospho-tyrosine pattern reflects moderate and severe signal transduction dysregulations in ILK-wk and ILK-hk mutants, respectively. These dysregulations are seen after the specific induction of $\beta 1$ -integrin clustering and suggest ILK as a critical regulator of $\beta 1$ -integrin membrane dynamics. The distinctive responses of, for example, AKT or MAPK in ILK-wk *versus* ILK-hk mutants were observed after irradiation and cannot be directly compared with $\beta 1$ -integrin-related responses. In case of a 2-Gy irradiation, the whole cell is diffusely damaged and/or stimulated, and interpathway compensation for survival is highly likely to occur. In contrast, $\beta 1$ -integrin stimulation is a very defined event and the modifications of these pathways seem to be because of ILK's role in the regulation of engagement and disengagement of various downstream molecules. The increasing evidence for intensive mutual cross-talk between integrin cascades with growth factor receptor signaling (18) has strongly to be considered for compensatory effects within cellular response patterns after genotoxic injury.

Besides impacting on $\beta 1$ -integrin clustering, the presented data support a role of ILK in the regulation of conformational changes of $\beta 1$ -integrins, which render critical for adhesion events (13). Interestingly, adhesion to fibronectin, vitronectin, or collagen-III was significantly reduced in ILK-hk but was unaffected in ILK-wk mutants. Blocking experiments showed equipotent effects in ILK mutants and empty vector controls. Thus, overexpression of ILK seems to nega-

¹ N. Cordes, unpublished observations.

tively modify integrin affinity via inside-out signaling in an activity-dependent manner.

In summary, these data on ILK-mediated integrin signaling give additional evidence for an important regulatory role of cell-extracellular matrix-interactions in cellular radiation survival and proliferation responses. Overexpression of ILK, especially in its constitutively active form, disrupts normal cell physiology because of inhibitory interference with diverse signaling and structural molecules. The impact of enhanced radiosensitization of constitutively active ILK has to be proven in additional *in vitro* and *in vivo* studies. Identification of the underlying molecular mechanisms will possibly provide considerable insight into the understanding of cell adhesion-mediated radioresistance, oncogenic transformation, and tumor growth and spread. These insights could help to promote innovative anticancer strategies.

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