**Diacylglycerol kinase-α mediates HGF-induced epithelial cell scatter by regulating Rac activation and membrane ruffling.**

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**ABSTRACT**

Diacylglycerol kinases (Dgk) phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), thus turning off and on, respectively, DG-mediated and PA-mediated signalling pathways. We previously showed that HGF, VEGF and ALK activate Dgkα in endothelial and leukaemia cells through a Src-mediated mechanism, and that activation of Dgkα is required for chemotactic, proliferative and angiogenic signalling *in vitro*. Inhere we investigate the downstream events and signalling pathways regulated by Dgkα, leading to cell scatter and migration upon HGF treatment and v-Src expression in epithelial cells. We report that inhibition of Dgkα, obtained either pharmacologically or by expression of a dominant-negative mutant, impairs *i)* HGF- and v-Src-induced cell scattering and migration, without affecting the loss of intercellular adhesions; *ii)* HGF-induced cell spreading, lamellipodia formation, membrane ruffling and focal adhesions remodelling; *iii)* HGF-induced Rac activation and membrane targeting. In summary, we provide evidence that Dgkα, activated downstream of tyrosine kinase receptors and Src, regulates crucial steps directing Rac activation and Rac-dependent remodelling of actin cytoskeleton and focal contacts in migrating epithelial cells.

## INTRODUCTION

Epithelial tissues are characterized by monolayers of highly polarized cells, while *in vitro* epithelial cells grow to form discrete colonies. During embryonic development and tissue repair, as well as through cancer progression, epithelial cells acquire a highly motile and invasive phenotype in a process commonly known as epithelial-mesenchymal transition (EMT) (Thiey, 2002; Thiery *et al.*, 2006). *In vitro*, the scattering of epithelial cells, *i.e.* the dispersal of colonies due to loss of intercellular adhesion and acquisition of cell motility, is triggered by growth factors stimulation and by oncogenes activation, recapitulating the early phases of EMT (Avizienyte and Frame, 2005).

HGF and oncogenic Src induce *in vitro* cell scatter of several epithelial cells, while *in vivo* their inappropriate activation is associated to progression and acquisition of a metastatic phenotype in several epithelial-derived cancer (Irby and Yeatman, 2000; Danilkovitch and Zbar, 2002). Within hours from stimulation of their tyrosine kinase activities, both HGF and v-Src induce scattering of epithelial cell colonies through loss of cadherin-mediated cell-cell adhesions and increase of their motility, due to formation of lamellipodia and remodeling of cortical actin and focal adhesions (Beherens *et al.*, 1993; Lamorte *et al.*, 2002). The signalling pathways by which HGF and v-Src stimulate EMT, cell scattering and invasiveness have been extensively investigated in several epithelial cells (Thiery, 2002). Recruitment of Gab-1, along with activation of PI 3-kinase, PLCγ, Ras and Rac are required (Lamorte *et al.*, 2002 and refs herein). Src plays a crucial role in HGF signalling as its activity is required for HGF-mediated cell motility, anchorage-independent growth and tumorigenesis. Indeed Src mediates HFG-induced tyrosine phosphorylation of catenins, leading to downregulation of cadherin-mediated cell-cell adhesions, and of several focal adhesion proteins required for cell motilitiy and invasiveness, such as FAK, Paxillin and p130Cas (Beherens *et al.*, 1993; Rahimi *et al.*, 1998; Nakaigawa *et al.*, 2000).

Diacylglycerol kinases, which phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), comprise a family of ten distinct enzymes, grouped in five classes each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich atypical C1 domains (Topham and Prescott, 1999; Imai *et al.*, 2005). DG is an established activator of several typical C1 domain-containing proteins, such as PKCs, RasGRPs and chimaerins. Similarly, several signalling proteins have been reported to be regulated by PA, including serine kinases, such as mTor, Raf and atypical PKCs, small GTPase regulating proteins, such as RhoGDI, Ras- and Rho-GAPs, and signalling lipid metabolizing enzymes, such as PI4P 5-kinase and PLC-γ (Topham, 2006). However, a common specific PA binding domain has not been identified yet. Thus, by

regulating in a reciprocal manner the level of both DG and PA lipid second messengers, Dgk enzymes may act as terminators of DG-mediated signals as well as activators of PA-mediated ones.

Recent evidences showed that α, ζ and θ Dgk isoforms are regulated by extracellular ligands and play a role in signal transduction (van Blitterswijk and Houssa, 2000; Luo *et al.*, 2003). T cells derived by Dgkα -/- mice feature enhanced DG-mediated RasGRP activity upon TCR activation, leading to over-activation of the Ras pathway and a defect in anergy, while overexpression of Dgkα in T cells impairs TCR signalling (Olenchock *et al.*, 2006). Several evidence in T cells indicate that Dgkα and ζ, by interacting respectively with RasGRP and PKC, up-regulate cell sensitivity to TCR activation by negatively modulating the intensity and the kinetic of DG-mediated signalling (Luo *et al.*, 2003; Sanjuan *et al.*, 2003; Zhong *et al.*, 2003). Conversely, mast cells derived from Dgkζ -/- mice feature a diminished FcεRI-mediated degranulation, correlating with impaired PLCγ activation and calcium response, both likely dependent on PA production (Olenchock *et al.*, 2006).

We have previously shown that in endothelial and leukaemia cells, activation of Dgkα downstream from tyrosine kinase receptors, such as HGF-R, VEGFR-2, and ALK, is required for either chemotactic or proliferative signalling induced by their respective ligands, as well as for cell proliferation upon IL-2 stimulation of T cells (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004; Bacchiocchi *et al.*, 2005). Growth factors stimulate Dgkα through a mechanism requiring complex formation with Src and tyrosine phosphorylation of Dgkα by Src itself. The specific signalling pathways regulated by activation of Dgkα still await elucidation.

Herein we investigate the role of Dgkα in HGF-induced cell migration of epithelial cells. We show that Dgkα activation is required for HGF- and v-Src-induced scattering of MDCK cells, and particularly in those mechanisms leading to cell spreading and F-actin cytoskeleton and focal adhesions remodelling. By further investigating the role of Dgkα in HGF early signalling, we show that upon 15 minutes from HGF stimulation, Dgkα activity is necessary for membrane targeting and activation of Rac, and for Rac-regulated formation of membrane ruffles.

Thus these data, by indicating Dgkα as a key signal transducer of motility signals downstream HGF and v-Src, strongly suggest that it may represent a key regulator in the processes of invasion and metastasis.

## MATERIALS AND METHODS

### *Cell culture*

MDCK (Madin-Darby canine kidney cells) and MDCK stably expressing ts-v-Src (4) are a kind gift of W. Birchmeier (Berlin). Cells were cultured in high glucose DMEM medium (Sigma), supplemented with 10% fetal bovin serum (Gibco) and antibiotic-antimicotic solution (Sigma), in humidified athmosphere with 5% $CO_2$. MDCK cells were cultured at 37°C, while MDCK-ts-v-Src were normally grown at 40.5°C (inactive Src) and shifted to 35°C to achieve Src activation.

### *Reagents*

Recombinant human HGF was purchased from Peprotech; R59949 (Diacylglycerol Kinase Inhibitor II) from Sigma. DMSO, vehicle for R59949, was always used in control samples at the same dilution of R59949.

Anti-Myc clone 9E10, and anti-Rac1 were from Upstate, anti-Paxillin from BD Transduction Laboratories, anti-Paxillin $pY^{31}$ and $pY^{118}$ and anti-Akt $pS^{473}$ from Biosource, anti-Akt from Cell Signling, anti-α-Tubulin from Sigma, anti-Vinculin from Novus Biological, anti-FAK from Calbiochem, Alexa Flour 546 Phalloidin from Molecular Probes. Secondary HRP-conjugated antibodies were purchased from PerkinElmer Life Sciences; Secondary FITC- and TRITC-conjugated antibodies were purchased from DAKO.

### *Expression vectors, transfections and infections with retroviral vectors*

Myc-Dgkα cDNA cloned into pMT2 expression vector have been previously described (Cutrupi *et al.*, 2000). GFP-Dgkα-WT was obtained by cloning Dgkα-WT in pcDNA-DEST53 (Invitrogen) using the Gateway kit (Invitrogen) according to manufacturer's instructions. Briefly, Dgkα-WT cDNA was inserted in pDONOR 2.11 vector by PCR and BP recombination. LR recombination was performed to transfer Dgkα-WT in the Invitrogen vector pcDNA-DEST53 for N-terminal GFP fusion; detailed information and protocols are available on www.invitrogen.com. $G_{434}D$ point mutation on Dgkα to obtain the kinase-defective mutant (GFP-Dgkα-DN) was performed using QuikChange Site-Directed Mutagenesis Kit 22 (Stratagene) as previously described (11). PINCOS retroviral vector and PINCOS/Dgkα-DN have already been described (11).

Transient transfections were performed using Lipofectamine2000 Reagent (Invitrogen) according to the manufacturer's instructions.

MDCK and MDCK-*ts*-v-Src stably expressing PINCOS vector alone or PINCOS/Dgkα-DN were obtained by infection of retroviral vectors. Briefly, GP2-293 packaging cell line (Clontech,

kindly provided by R. Piva, CERMS, Turin, Italy) was transiently co-transfected, by Lipofectamine2000 Reagent (Invitrogen) according to the manufacturer's instructions, with the envelope vector pVSV-G (Clontech) together with PINCOS or PINCOS/Dgkα-DN in Opti-MEM (Invitrogen). The next day the medium was changed with DMEM 10% FBS growth medium. After 48 hours from infection, the retroviral supernatant was collected, the debris removed by centrifugation at 1500g, filterd by a 0.45 μm pore filter and added with Polybrene (8 μg/ml). MDCK cells, plated in a six-well plate, were infected by addition of 2 ml retroviral supernatant and 1 ml of growth medium. The next day after this first infection cells were re-infected as before. Sixteen hours later, cells were placed and maintained in growth medium. Efficiency of infection was about 80% as measured by FACS analysis and/or observation with fluorescence microscope of cells expressing GFP.

***Dgk assay***

Dgkα activity was assayed in anti-Myc immunoprecipitates essentially as described (Cutrupi *et al.*, 2000). Briefly, after immunoprecipitation and extensive washing in Lysis Buffer, Litium Cloride Buffer (500 mM LiCl, 25 mM Tris-HCl pH 8) and TNE (Tris 25 mM pH 8, NaCl 150 mM, EDTA 1 mM), all supplemented with fresh fresh 1 mM $Na_3VO_4$, immunocomplexes were assayed at room temperature for 10 minutes by incubation with 0.3 mg/ml diolein (Fluka, dried in nitrogen athmosphere, resuspended and sonicated in 1 mM EGTA, 25 mM Hepes pH 8), 500 μM ATP, 10 μCi/sample [$\gamma$-$^{32}$P]ATP (Amersham), 10 mM $MgCl_2$, 1 mM $ZnCl_2$. Lipids were then extracted as described (15), and PA was separated by TLC in chloroform: methanol: water: 32% ammonium hydroxide (60:47:10:3). TLC plates had been previously coated with [potassium oxalate 1.3%, EDTA 5 mM]:methanol 3:2. [$^{32}$P]-PA was identified by co-migration with non-radioactive PA standards (Fluka) stained by incubation in iodine chamber. Radioactive signals were detected and quantified by GS-250 Molecular Imager and its Phosphor Analyst Software (Biorad). Usually, one half of immunoprecipitated lysates was assayed for Dgk activity, while the other half was heat-denatured in Laemmli Buffer, runned in SDS-PAGE and blotted for anti-Myc.

***Scatter and chemotaxis***

For HGF-induced cell scatter, MDCK were plated at low density in 24-wells plates and allowed to growth in small colonies. Cells were stimulated in serum-free medium with 1.25 ng/ml HGF for 24 hours in presence or absence of 1μM R59949, fixed with 3% paraformaldheyde, 4% sucrose in PBS, and then photographed with phase-contrast optics with a 20x objective (Zeiss). .

For v-Src induced cell scatter, MDCK-*ts*-v-Src cells were overnight shifted to the permissive temperature of 35°C in DMEM 2% FBS in presence or absence of 1 μM R59949.

Chemotaxis assays were performed in a Neuro Probe Standard 48 Well Chemotaxis Chamber according to manufacturer'instructions. Briefly, the bottom chamber was filled with serum-free DMEM containing 50 ng/ml as chemoattactant, in presence or absence of 1μM R59949. $10^5$ cells were seeded in the upper chamber and let to migrate overnight through a polycarbonate filter coated with 0.1% gelatine. Migrated cells were fixed and stained with Diff-Quick (Dade Behring Inc.) before counting.

***Invasion***

Invasion assays were performed in serum-free medium in 6.5 mm Transwells with 8 μm-pore size membranes. The Transwell membrane was pre-coated with 10 μg Matrigel (BD Biosciences) in 50 μl of cold serum-free medium and dried overnight at room temperature. $10^5$ MDCK cells were seeded in the upper chamber of the Transwell apparatus. The lower chamber was filled with DMEM 2% FBS in presence or absence of 100ng/ml HGF and cells were allowed to migrate for 48 hours. After washing with PBS, the cells on the upper surface of the Transwell membrane were then removed using a cotton-tipped swab, while those cells onto the lower face were fixed in glutaraldehyde and stained with crystal-violet. Fixed cells were then photographed and invasion was quantified by optical densitometry**.**

***Immunofluorescence***

MDCK cells were seeded in small colonies on glass coverslips (Marienfeld) in 24-well cell culture plates. Cells were overnight starved and then stimulated with 10 ng/ml HGF for the indicated times. 1μM R59949 was given as pre-treatment in short-time HGF experiments (15 minutes), while in long-time experiments (from 4 hours onward) was given together stimulus. After stimulation, cells were washed twice in PBS and fixed by incubation with PBS 3% paraformaldheyde-4% sucrose. After washes in PBS, cells were permeabilized with a cold HEPES-Triton Buffer (20 mM HEPES pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100). Cells were then washed with PBS containing 0.2% BSA and incubated for 15 minutes with PBS containing 2% BSA. 15 μl of primary antibody (1:100 in PBS/2% BSA) was added directly onto each glass coverslip in a humidified chamber for 30 minutes and the excess of antibody was washed away with PBS/0.2% BSA. Cells were then incubated for additional 15 minutes with PBS/2% BSA and FITC- or TRITC-conjugated secondary antibodies and/or Alexa Fluor 546 Phalloidin (1:30 and 1:200 in PBS/2% BSA respectively) were added for 30 minutes in the

humidified chamber. After washes, each glass coverslip was washed briefly in water and blocked onto a glass microscope slide by Mowiol (20% Mowiol 4-88, 2.5% DABCO in PBS 1X pH 7.4) and let polymerize. Confocal images were acquired with the Leica confocal microscopy TSP2 and relative software. Basal planes are shown.

***Western blotting***

Cell lysates were prepared after cold-PBS washing by scraping on ice in Lysis Buffer (25 mM HEPES pH 8, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 10% glycerol supplemented with fresh 1 mM $Na_3VO_4$ and protease inhibitors [Protease Inhibitors Cocktail, Sigma]). Clarified lysates were denatured by boiling in Laemmli Buffer. For Paxillin detection, 10% polyacrylammide gel was used.

***RacGTP pull-down assay***

RacGTP pull-down assays were performed according to Zondag et al. (69). Briefly, MDCK cells were seeded in 15 cm-diameter cell culture plates and overnight starved in 0% FBS DMEM before stimulation with 100 ng/ml HGF for 15 minutes. 1μM R59949, when used, was added with a 15 minutes pretreatment and maintained during the following HGF stimulation. Cells were then washed in ice-cold PBS and lysed with GST-Fish Buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% glycerol, 0.1% Triton X-100 supplemented with fresh 1 mM $Na_3VO_4$, protease inhibitors and 1 mM DTT) and harvested by scarping. The clarified lysates were incubated for 45 minutes at 4°C with purified GST-PAK-BD, pre-coupled to GSH-Sepharose beads (GE Healthcare). After 3 washes with GST-Fish Buffer, samples were resuspended in Laemmli Buffer, heat-denatured and separated by SDS-PAGE in a 15% polyacrylammide gel. A small amount of each sample was directly denatured in Laemmli Buffer for whole cell lysates proteins analysis (Rac, phospho-Akt, Akt, tubulin). Western Blot bands of active and total Rac were analyzed by optical densitometry (QuantityOne, Biorad) and the ratio GTP-bound Rac/total Rac was calculated. Activation was expressed as percentage of activation respect to HGF-treated cells.

## RESULTS

### *Dgkα activation mediates HGF-induced scatter and migration of MDCK cells.*

We already showed that activation of Dgkα in endothelial cells is required for VEGF and HGF-induced chemotaxis (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004). However the role of Dgkα in epithelial cell scattering has never been investigated, as well as the signalling pathways involved.

MDCK cells express endogenous Dgkα and feature a R59949-sensitive Dgk activity associated to anti-phosphotyrosine immunoprecipitates upon HGF stimulation (data not shown). Upon v-Src activation, obtained by shifting MDCK-*ts*-v-Src cells to the permissive temperature, Dgkα is activated in a time-dependent manner reaching a maximum activity after 1h (Figure 1). Activation of Dgkα was evaluated by immunoprecipitation of a Myc-tagged form of Dgkα (Myc-Dgkα), transiently transfected in MDCK cells, as no appropriate antibodies are available for canine Dgkα. Similarly, Myc-Dgkα was also activated by HGF in MDCK cells (data not shown), as previously reported in endothelial cells (Cutrupi *et al.*, 2000).

MDCK cells form discrete compact colonies that, upon either HGF stimulation or v-Src activation, undergo scatter, which involves cell spreading, dissolution of inter-cellular adhesions and migration of cells away from each other (Beherens *et al.*, 1993; Weidner *et al.*, 1993; Palacios and D'Souza-Schorey, 2003).

In order to investigate the role of Dgkα in cell scattering and migration, Dgkα activity was inhibited in MDCK cells either by treatment with R59949, an isoform-specific Dgk inhibitor or by stable expression of Dgkα kinase-defective mutant, acting as dominant-negative (Dgkα-DN) (Cutrupi *et al.*, 2000). Both cell treatment with 1μM R59949 (Figure 2A) and expression of Dgkα-DN (Figure 2B), severely impair HGF-induced cell scatter. Similarly, inhibition of Dgkα, either by R59949 or Dgkα-DN, strongly impairs MDCK cells scattering induced upon v-Src-*ts* activation (Figure 2C). We observed that inhibition of Dgkα, while impairing full cell scattering, does not affect either HGF- or v-Src-induced down-regulation of cell-cell adhesions. This observation suggests that activation of Dgkα is not required for HGF- and v-Src-induced down-regulation of cell-cell adhesions, but is specifically involved in the signalling pathways leading to cell migration. We further verified this hypothesis in a quantitative chemotaxis assay. Indeed 1μM R59949 completely abolishes HGF-induced chemotaxis of MDCK cells toward the HGF-filled lower chamber (Figure 2D), while it does not affect cell basal migration.

A motile phenotype is essential also for the acquired ability of scattering MDCK cells to invade the extracellular matrix, a typical feature of metastatic carcinoma. Thus we verified the role

of Dgkα in HGF-induced invasion of MDCK cells through a Matrigel barrier, a common assay to investigate the signalling pathways leading to metastatic progression (Birchmeier *et al.*, 2003). Indeed inhibition of Dgkα strongly impairs HGF-induced *in vitro* invasiveness of MDCK cells (Figure 2E).

***Dgkα inhibition uncouples spreading, cytoskeletal remodelling and lamellipodia formation from down-regulation of E-cadherin-mediated intercellular adhesions.***

In HGF-induced cell scattering, loss of cell-cell contacts is preceded by internalization of E-cadherins at 4-6 hours from HGF stimulation (Beherens *et al.*, 1993; Potempa *et al.*, 1998; Kimura *et al.*, 2006), which occurs concomitantly to colony spreading, so that the area covered by each colony increases two to three fold. At the same time, cells at the colony's outer edge undergo dramatic morphological changes, featuring extended lamellipodia, where focal adhesion proteins, such as Paxillin and Focal Adhesion Kinase (FAK), are recruited at new sites of adhesion and newly-formed stress fibres (Weidner *et al.*, 1993; Ridley *et al.*, 1995; Palacios and D'Souza-Schorey, 2003).

We observed that inhibition of Dgkα, either by 1μM R59949 treatment (data not shown) or by expression of Dgkα-DN, does not affect the internalization and removal of E-cadherins from cell-cell contacts (Figure 3A, panel j), occurring upon 6 hours of HGF treatment. Conversely, inhibition of Dgkα by 1μM R59949 results in a remarkable reduction of colony spreading (Figure 3B) induced upon 4 hours of HGF treatment. Moreover, staining for F-actin clearly shows that Dgkα inhibition strongly affects HGF-dependent morphological changes such as lamellipodia formation (Figure 3C, panels a,d,g,j). Consistently with inhibition of lamellipodia formation, R59949 treatment severely impairs HGF-induced recruitment of focal adhesion proteins, such as Paxillin, Vinculin (data not shown) and FAK, at newly-formed adhesion sites (Figure 3C, panels b,e,h,,k). Inhibition of Dgkα in unstimulated MDCK cells does not affect their morphology concerning all of the analyzed aspects.

These data strongly suggest that Dgkα is not involved in the mechanisms by which HGF and v-Src downregulate E-cadherin-mediated intercellular adhesions, and that its inhibition uncouples HGF-induced events leading to loss of intercellular adhesions, from signalling pathways mediating cell spreading, F-actin remodelling, lamellipodia formation and eventually cell migration.

***Dgkα is required for HGF-induced membrane ruffles formation and focal complex remodelling***

Upon few minutes from HGF stimulation, cells at the outer edge of colonies undergo intense

ruffling. They eject small membrane protrusions, whose formation relies on regulated recruitment of molecular scaffolds to growing focal complexes at new adhesion sites, coupled to the coordinated organization of actin filaments into lamella network and bundled arrays. Eventually membrane ruffles evolve in wider lamellipodia driving and providing direction to cell migration (Small *et al.*, 2002). Thus we verified whether the effects of Dgkα inhibition observed after hours of HGF stimulation derived from impairment of events occurring at earlier time points, such as formation of membrane ruffles and new focal complexes.

We determined Dgkα localization in resting or HGF-treated MDCK cell by transiently transfecting a GFP-Dgkα fusion protein.. In untreated cells Dgkα displays cytoplasmic localization, but upon 15 minutes of HGF treatment it accumulates at the cell periphery, in correspondence of the protruding plasma membrane (Figure 4A). This observation suggests that Dgkα may play a role in HGF-induced earlier events leading to membrane ruffle formation.

Thus we set to investigate earlier changes in F-actin cytoskeleton organization in response to HGF. Upon 15 minutes of HGF treatment, small membrane ruffles develop on the outer membranes of cells at colony edge (Figure 4B, arrow). The percentage of cells featuring membrane ruffles raises from less than 20% in control cells (vehicle alone or R59949-treated cells) to about 50% in HGF-treated cells. In presence of 1μM R59949, the percentage of membrane ruffles-displaying cells upon HGF stimulation is reduced to almost control value (Figure 4C). Consistently, HGF fails to induce membrane ruffles in cells expressing Dgkα-DN compared to cells expressing the vector alone (Figure 4D and 4E). In conclusion, these data demonstrate that the formation of membrane ruffles occurring upon 15 minutes of HGF stimulation, depends on stimulation of Dgkα activity.

Membrane ruffles formation implies the recruitment of focal adhesion proteins at new adhesion sites within the ruffle itself. In epithelial cells, Paxillin recruitment to newly-formed focal complexes, where it acts as a scaffold for signalling complexes, is required for HGF-induced signalling leading to cell migration (Lamorte *et al.*, 2003; Ishibe *et al.*, 2004; Chen *et al.*, 2005).

In resting MDCK cells, Paxillin is partially diffuse in the cytoplasm, while in cells at colony edge it is also localized in focal adhesions along the outer plasma membrane (Figure 5A and B, panel a). Upon 15 minutes of HGF stimulation, Paxillin condensates to the newly-formed focal complexes in correspondence to membrane ruffles (Figure 5A, panel g and Figure 5B, panel d). Conversely, upon inhibition of Dgkα by either 1μM R59949 (Figure 5A, panel j), or by expression of Dgkα-DN (Figure 5B, panel j), Paxillin accumulates at peripheral focal adhesions along the outer plasma membrane, while ruffles formation is impaired. Inhibition of Dgkα in unstimulated cells does not significantly affect Paxillin localization either in the cytoplasm or at focal adhesions along the outer plasma membrane.

In order to verify that Paxillin indeed accumulates at focal complexes, we analyzed its co-localization with Vinculin, a resident protein of focal complexes, whose function is to stabilize them (Ziegler *et al.*, 2006). In unstimulated cells Vinculin and Paxillin co-localize at focal complexes along the outer membrane of cells at colony edge and upon HGF stimulation they are both recruited to newly-formed focal complexes, in a manner fully dependent on Dgkα activity. In fact, inhibition of Dgkα, while impairing HGF-induced neo-formation of focal complexes at membrane ruffles, does not affect Vinculin and Paxillin co-localization (Figure 5C).

Upon growth factor stimulation Src- and FAK-mediated phosphorylation of Paxillin is required to recruit and coordinate multiple signalling complexes, regulating events at the leading edge of the migrating cells (reviewed in Brown and Turner, 2004). Phosphorylation of Paxillin on tyrosine 31 and 118 mediates its association with Crk and is required for growth factors-induced Paxillin-mediated migratory signals (Nakamura *et al.*, 2000; Petit *et al.*, 2000). Thus we verified whether inhibition of Dgkα affects HGF-induced phosphorylation of $Tyr^{31}$ and $Tyr^{118}$, identified by anti-phosphotyrosine specific antibodies, both in immunofluorescence and western blot. Phosphorylation of Paxillin on both $Tyr^{31}$ and $Tyr^{118}$ was detected in intact cells by staining with a 1:1 mixture of the respective specific antibodies (Chem *et al.*, 2004). Figure 5D clearly shows that upon 15 minutes HGF stimulation, Paxillin is recruited to the newly formed focal complexes at membrane ruffles and is heavily phosphorylated on both $Tyr^{31}$ and $Tyr^{118}$. Interestingly, inhibition of Dgkα, while severely impairs HGF-induced formation of membrane ruffles and of new sites of adhesion, as described above, does not appear to affect Paxillin phosphorylation on $Tyr^{31}$ and $Tyr^{118}$, either at basal level in unstimulated or in HGF-stimulated cells. These observations are further supported by western blot analysis of Paxillin tyrosine phosphorylation. In fact HGF-induced phosphorylation of neither $Tyr^{31}$ nor $Tyr^{118}$ was affected upon inhibition of Dgkα (Figure 5E).

In summary these data demonstrate that upon minutes from HGF stimulation, activation of Dgkα is required for formation of membrane ruffles and for the succeeding remodelling of Paxillin- and Vinculin-containing focal complexes, but is not required for HGF-induced signalling leading to Paxillin tyrosine phosphorylation.

***Dgkα is required for HGF-induced Rac activation and membrane targeting .***

The data presented above strongly suggest that activation of Dgkα is involved in the signalling mechanisms leading from HGF-receptor activation to ruffle formation.

Membrane ruffle formation is dependent on activation of Rac small GTPase, which acts upstream of the recruitment of WAVE and Arp2/3 complexes at new adhesion sites promoting F-

actin polymerization (Takenawa *et al.*, 2007). In migrating cells active Rac localization at leading edge is enhanced and allows the coupling with its downstream effectors (Kurokawa and Matsuda, 2005). In MDCK cells HGF activates Rac, whose function is required for HGF-induced cell scatter, spreading, ruffles and lamellipodia formation (Ridley *et al.*, 1995; Royale *et al.*, 2000).

Activation of endogenous Rac was assayed by GST-PAK pull-down to purify active GTP-bound Rac from lysates of either control or HGF-stimulated MDCK cells. HGF treatment results in activation of endogenous Rac. Inhibition of Dgkα, by either 1μM R59949 or by expression of Dgkα-DN, severely impairs HGF-induced Rac activation, without affecting Rac basal state of activation (Figure 7A and 7B).

Rac activation is tightly coupled to its targeting to specific cholesterol-enriched membrane microdomains, defined by ligand-activated integrin signalling (Grande-Garcìa *et al.*, 2005). Thus we verified whether inhibition of Dgkα may interfere with HGF-induced targeting of Rac to the plasma membrane. By confocal microscopy, we observed the localization of endogenous Rac in MDCK cells (Figure 8A and 8B). In most unstimulated cells Rac is both cytoplasmic and at intercellular contacts, while only about 20% of cells at colonies edge feature Rac at the outer plasma membrane (Figure 8A, panel a). Following 15 minutes of HGF stimulation, the number of colony-edge cells featuring Rac at the outer cell membrane raises to more than 40% (Figure 8A, panel g and 8B), while localization of Rac at cell-cell contacts is not affected. Inhibition of Dgkα by 1μM R59949 treatment completely abolishes HGF-induced Rac membrane targeting (Figure 8A, panel j and 8B), while it does not significantly affect Rac localization in unstimulated cells (Figure 8A, panel d), nor Rac localization at cell-cell contacts. Similar results were obtained when Dgkα was inhibited upon expression of Dgkα-DN (Figure 8C and 8D). Upon HGF stimulation Rac is properly membrane localized in cell infected with the vector alone (Figure 8C, panel d, thick arrow) and in uninfected cells (Fig. 8C, slim arrows), while it remains predominantly cytoplasmic in Dgkα-DN-expressing cells (Fig. 8C, panel j).

In summary these data demonstrate that Dgkα is required for HGF-induced activation and targeting of Rac to the plasma membrane and for the following formation of membrane ruffles. Thus these observations strongly suggest that Dgkα is involved in the signalling pathways regulating Rac function and targeting upon activation of HGF receptor. Thoroughly, this data demonstrate that Dgkα plays a pivotal role in the migratory signalling downstream HGF, being involved in early molecular events such as Rac activation, membrane ruffles protrusion and formation of new focal adhesions, and that it consequently regulates the acquisition of a migratory phenotype in epithelial cells.

## DISCUSSION

In this study we investigated the role of Dgkα in HGF- and v-Src-induced cell migration. We show that Dgkα inhibition, obtained either pharmacologically or by expression of a kinase-defective dominant-negative mutant, impairs both HGF- and/or v-Src-induced cell scatter and migration. This finding is consistent with previous demonstrations from our laboratory that Dgkα is activated by growth factors through a mechanism requiring its tyrosine phosphorylation mediated by Src family tyrosine kinases, and that its function is required for migration of endothelial cells (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004; Bacchiocchi *et al.*, 2005). Moreover these data suggest that Dgkα represents a crucial node in the signalling network downstream Src regulating epithelial cell scattering and switching to a motile mesenchymal phenotype.

Although both HGF stimulation and v-Src activation promote epithelial cell dispersion by coordinating loss of intercellular adhesions and migration of cells away from each other, the two events are regulated through distinct signalling pathways (Palacios *et al.*, 2001). Intriguingly, Dgkα inhibition uncouples the down-regulation of E-cadherins-mediated intercellular adhesions from cell migration, strongly suggesting that Dgkα may regulate specifically those signalling events required for HGF- and v-Src-stimulated epithelial cell motility. Thus we investigated the role of Dgkα in well characterized HGF-induced morphological and molecular events leading to cell migration.

Spreading and lamellipodia protrusion with formation of new focal adhesions at the leading edge are mandatory steps in cell migration (Ridley *et al.* 1995; Small *et al.*, 2002). We show inhere that upon Dgkα inhibition no cell spreading, lamellipodia extension and remodelling of focal adhesions are observed upon HGF treatment, suggesting that activation of Dgkα is likely to be required for an earlier event. Rapid formation of membrane ruffles, upon minutes from growth factors stimulation, preludes to establishment of extended lamellipodia at the leading edge of migrating cells (Royale *et al.*, 2000). Indeed, upon inhibition of Dgkα, MDCK cells fail to extend membrane ruffles after HGF stimulation. Intriguingly, while recent findings indicate that Dgkα is enriched in the pseudopodia of spontaneously invasive epithelial MSV-MDCK-INV cells (Jia *et al.*, 2005), we show that Dgkα is recruited to membrane ruffles upon HGF treatment. Altogether these data provide the first circumstantial evidence that Dgkα may act in growth factors signalling at the leading edge of migrating cells.

Ruffles formation, cell spreading and lamellipodia protrusion are dependent on Rac small GTPase activation, occurring through its targeting to newly-formed focal complexes (Ridley *et al.*, 1995; Burridge and Wennerberg, 2004). Rac targeting and GTP loading are regulated by a complex signalling network involving the recruitment of distinct Rac-regulating proteins to multiple

molecular complexes at the leading edge of migrating cells.

An increasing body of evidence suggests that Dgks regulate small GTPases, including Rac, through multiple mechanisms, whose complexity still awaits elucidation. In T cells Dgkα and ζ negatively regulate Ras pathway, by finely tuning the access of RasGRP1, a C1 domain-containing Ras GEF, to its activator DG (Jones *et al.*, 2002; Olenchock *et al.*, 2006, Zha *et al.*, 2006). However, in epithelial cells, neither the over-expression nor the down-regulation of Dgkα affect the Ras pathway, as detected by ERK-1/2 phosphorylation (our unpublished results). In addition Dgkγ, but not Dgkα, upon its recruitment to the plasma membrane, negatively regulates PDGF- and EGF-induced Rac activation and membrane ruffling, by enhancing the activity of β2-chimaerin, a Rac GAP containing a C1 and a SH2 domain (Tsushima *et al.*, 2004, Yasuda *et al.*, 2007). These observations provide further support to previous finding that DG-dependent membrane recruitment of β2-chiamerin determines the extent and the kinetic of EGF-induced Rac activation. (Wang *et al.*, 2006). However the role of either DG or PA in membrane recruitment and activation of β2-chimaerin is still controversial (Caloca *et al.*, 2003; Canagarajah *et al.*, 2004)**.** Conversely, in neurons and skeletal myoblasts Dgkζ acts in a complex with Rac at specific sites of the plasma membrane and control the remodelling of F-actin cytoskeleton leading to neurite extension and membrane ruffle protrusion, possibly by facilitating Rac1 activation and/or localization to the cell surface (Abramovici *et al.*, 2003; Yakubchyk *et al.*, 2005). Furthermore Dgkζ and PI(4)P 5-kinase co-localize with F-actin at lamellipodia protrusions in epithelial cells (Luo *et al.*, 2004), where Dgk-generated PA is required for full activation of PI(4)P 5-kinase activity, consistently with a role of both lipid kinases in positive regulating Rac function. Interestingly a Dgk and a PI(4)P 5-kinase activities were found to associate in a complex with Rac and RhoGDI (Tolias *et al.*, 1998). RhoGDI forms a complex with Rac, keeping it in a cytosolic inactive GDP-bound form, and upon Rac activation it contributes to Rac targeting to specific sites at the plasma membrane (Moissoglu *et al.*, 2006). As Rac targeting implies the displacement of the interaction between Rac and RhoGDI, the finding that *in vitro* PA and PI(4,5)$P_2$ impair RhoGDI affinity for Rac (Chuang *et al.*, 1993; Ugolev *et al.*, 2006), raises the hypothesis that activation of the RhoGDI associated Dgk may allow the release of Rac from RhoGDI, and leads to speculate that also Dgkα may regulate Rac activation through this mechanism. Altogether, these data strongly indicate that distinct Dgk isoforms act as regulators of Rac membrane targeting and activation through multiple mechanisms, whose complexity still awaits to be elucidated.

Several Rac GEFs, such as Vav2, DOCK180/Elmo, βPIX and Tiam1, are regulated either directly or indirectly through Src-dependent tyrosine phosphorylation (Lamorte *et al.*, 2002; Servitja *et al.*, 2003; Santy *et al.*, 2005), and/or interaction with PI(3,4,5)$P_3$ (Welch *et al.*, 2003).

Although there is no direct evidence for a role of any Dgk isoform in the regulation of any Rac GEF, based on the observations reported inhere, we may discuss several hypothesis, providing a framework for further investigation..

Several data indicate that, upon growth factors and v-Src stimulation, rapid Rac-mediated membrane ruffling occurs through the recruitment of βPIX to Paxillin-containing focal complexes (Cotton *et al.*, 2007). Indeed βPIX mediates rapid ruffles formation upon PDGF, EGF and FGF treatment in different cell types (Lee *et al.*, 2001; Park *et al.*, 2004; Shin *et al.*, 2006), and the interaction between βPIX and Rac is necessary and sufficient for Rac recruitment to membrane ruffles and focal adhesions (ten Klooster *et al.*, 2006). Crk recruitment to tyrosine phosphorylated Paxillin contributes to βPIX localization to focal complexes (Lamorte *et al.*, 2003). However, Dgkα inhibition does not affect Paxillin phosphorylation on either $Tyr^{31}$ or $Tyr^{118}$, the two major determinants for Crk association, suggesting that Dgkα may not regulate Rac activation through βPIX recruitment to the leading edge.

Upon minutes from growth factors stimulation, βPIX recruitment and Rac activation are promoted by rapid GTP/GDP cycling of Arf6, suggesting that Arf6 plays a pivotal role in Rac-mediated membrane ruffling (ten Klooster *et al.*, 2006; Cotton *et al.*, 2007). Interestingly, several Arf GAPs are regulated by phospholipids, including PA (Randazzo *et al.*, 2000). Moreover PLD-induced production PA downstream of Arf6 is required for Arf6-dependent epithelial cell ruffling and migration (Santy and Casanova, 2001). Thus we may speculate that also Dgkα may contribute to regulate Arf6 function in coordinating Rac activation, focal adhesions remodelling and membrane ruffles formation.

Several Rac and Arf GEFs are regulated by $PI(3,4,5)P_3$, the product of PI 3-kinase. However we can rule out that Dgkα may mediate Rac activation by regulating $PIP_3$ synthesis, as inhibition of Dgkα does not affect HGF-induced activation of Akt, a major $PIP_3$ target. Conversely, the finding that $PIP_3$ might contribute to recruit and activate Dgkα (Ciprés *et al.*, 2003) allow to speculate that Dgkα might contribute to couple $PIP_3$ generation to the activation of one of the $PIP_3$-dependent Rac GEFs, such as Vav2 and Tiam1. However, the expression of a either wild-type or kinase-defective Dgkα in fibroblasts does not affect Vav2-mediated activation of Rac and formation of membrane ruffles upon PDGF stimulation (Liu and Burridge, 2000; Tsushima *et al.*, 2004). Moreover, The Rac GEF Tiam1 is mainly involved in maintaining E-cadherins-mediated cell-cell adhesions of epithelial cells (Mertens *et al.*, 2003), an event which is not regulated by Dgkα, making Tiam1 an unlike target of Dgkα activity.

In conclusion, inhere we clearly demonstrate that activation of Dgkα is required for HGF- and v-Src-induced cell migration. By exploring some significant molecular events affected by Dgkα inhibition, we raise the hypothesis that Dgkα may act in growth factors migratory signalling by mediating Rac targeting and activation, thus revealing a novel signalling pathway linking RTKs and Src tyrosine kinase to small GTPases in the context of cell migration.

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## FIGURE LEGENDS

**Figure 1.** v-Src activates Dgkα. MDCK-ts-v-Src maintained at the non-permissive temperature of 40.5°C were transiently transfected with Myc-Dgkα, starved overnight in 0% FBS medium and shifted to the permissive temperature of 35°C for the times indicated. Cell lysates were immunoprecipitated with an anti-Myc antibody. Half of the immunoprecipitate was separated by SDS-PAGE and after blotting was probed with anti-Myc; the other half was assayed for Dgk activity as described in Materials and Methods.

**Figure 2.** Dgkα is required for HGF-induced cell scatter and migration of MDCK cells (A) MDCK cells colonies were treated, in 0% FBS medium, with HGF 1.25 ng/ml in presence or absence of 1μM R59949 for 24 hours. Representative fields are shown, photographed with a 20x objective. (B) PINCOS-infected or PINCOS/Dgkα-DN-infected MDCK cells were treated, in 0% FBS medium, with HGF 1.25 ng/ml for 24 hours. Representative fields are shown, photographed with a 20x objective. (C) PINCOS-infected or PINCOS/Dgkα-DN-infected MDCK-ts-v-Src cells colonies, maintained at the non-permissive temperature of 40.5°C, were serum starved in 0.2% FBS medium and shifted to the permissive temperature of 35°C, in presence or absence of 1μM R59949 for about 16 hours. Cells were then observed and photographed with phase-contrast optics. Representative pictures are shown. (D) MDCK cells were seeded in the upper part of a chemotaxis chamber and induced to migrate in presence of 50 ng/ml of HGF in the bottom part, in presence or absence of 1μM R59949. Histograms represent the number of migrated cells, means of 8 different wells with standard errors. ** = $p<0.005$. A representative experiment is shown. (E) PINCOS- or PINCOS/Dgkα-DN-infected MDCK cells were seeded in the upper chamber of a Transwell apparatus. Invasion through a Matrigel-covered porous membrane was induced in 48 hours in 0.2% FBS medium by the presence of 100 ng/ml HGF in the lower chamber. Fixed cells on the Transwells' lower face were stained with crystal-violet, photographed and quantified by optical densitometry. Means of three experiments are shown, with standard errors; * = $p \leq 0,05$.

**Figure 3.** Dgkα is required for HGF-induced spreading and lamellipodia formation, but not for down-regulation of E-cadherin-mediated intercellular adhesions. (A) PINCOS or PINCOS/Dgkα-DN-infected MDCK were treated with HGF 2.5 ng/ml for 6 hours, fixed and stained for E-cadherin. Representative pictures are shown. Scale bar = 20 μm. (B) MDCK cells colonies were starved overnight in a 2% FBS medium and treated with HGF 10 ng/ml for 4 hours, in presence or absence of 1μM R59949. Fixed cells were stained for actin filaments with

phalloidin.. Scale bar = 40 μm. (C) MDCK cells treated as described in (B) were fixed and stained for actin (red, panels a,d,g,j) and FAK (green, panel b,e,h,k). Scale bar = 16 μm. Representative pictures are shown

**Figure 4.** Dgkα is required for HGF-induced membrane ruffling of MDCK cells. (A) MDCK cells were transiently transfected with GFP-Dgkα, starved overnight in a 0% FBS medium, stimulated with HGF 50 ng/ml for 15 minutes, fixed and stained for actin. Scale bar = 8 μm (B) MDCK cells colonies were starved overnight in a 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for actin. Scale bar = 16 μm. (C) Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles (arrow). Percentage of cells with membrane ruffles was calculated. Means of three experiments with standard deviation are shown. ** = $p<0.005$. (D) PINCOS or PINCOS/Dgkα-DN-infected MDCK cells were treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for actin (red, panels a,d,g,j). Panels b,e,h,k show infected cells (green). The arrows indicate membrane ruffles in PINCOS-infected cells. Scale bar = 16 μm. Representative pictures are shown. (E) Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles. Means of three experiments with standard deviations are presented. * = $p<0.05$.

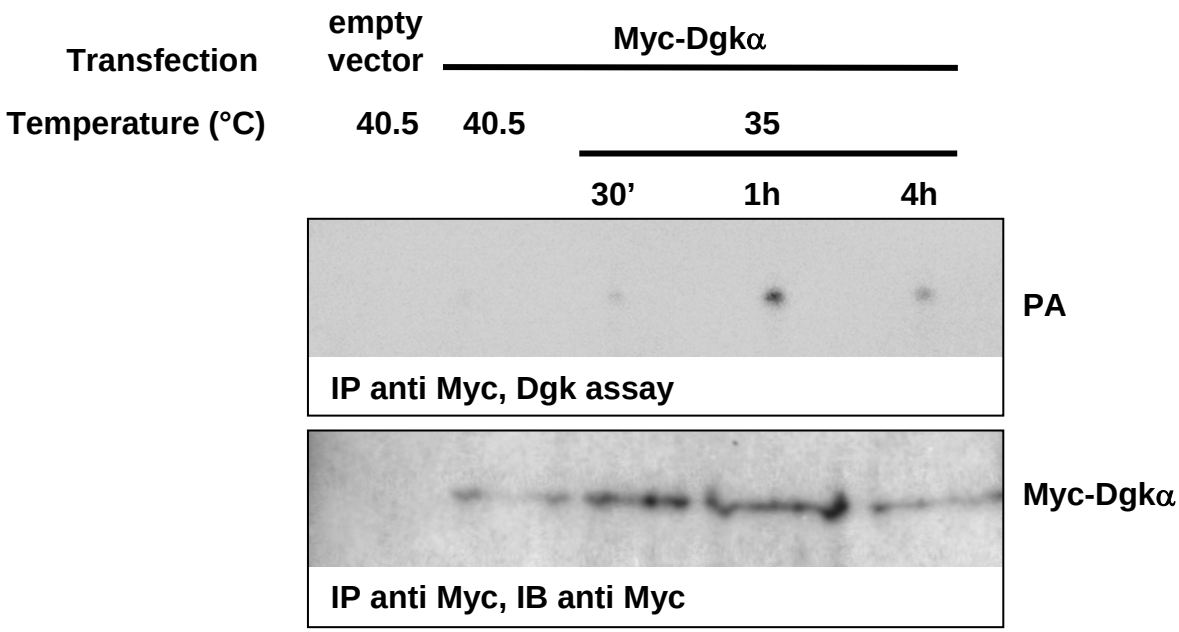
**Figure 5.** Dgkα is required for HGF-induced Paxillin localization to newly-formed focal adhesion. (A) MDCK cells colonies were starved overnight in a 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for Paxillin (green, panels a,d,g,j) and actin (red, panels b,e,h,k). Representative pictures are shown. Scale bar = 16 μm. (B) PINCOS or PINCOS/Dgkα-DN-infected MDCK cells were starved overnight in a 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for Paxillin (red, panels a,d,g,j). Panels b,e,h,k show infected cells (green). Thick arrows indicate Paxillin localization at focal adhesions at the sites of membrane ruffling, while the slim arrow indicates Paxillin localization at cell periphery in a Dgkα-DN-infected cell, without membrane ruffles. Scale bar = 16 μm. Representative pictures are shown. (C) MDCK cells colonies were treated as described in (A), fixed and stained for Paxillin (green, panels a,d,g,j) and Vinculin (red, panels b,e,h,k). Representative pictures are shown. Scale bar = 16 μm. (D) MDCK cells colonies were treated as in (A), fixed and stained for Paxillin $pY^{31}$:$pY^{118}$ 1:1 (green, panels a,d,g,j) and Paxillin (red, panels b,e,h,k). Representative pictures are shown. Scale bar = 16 μM. Nuclear staining in

panels a,d,g,j represent an aspecific signal. (E) MDCK cells were starved overnight in a 0% FBS medium and treated with HGF 100 ng/ml for 15 minutes, in presence or absence of 1μM R59949. Whole cell lysates were separated by electrophoresis, blotted and probed with anti-Paxillin $pY^{31}$ and $pY^{118}$.

**Figure 6.** Dgkα is required for HGF-induced Rac activation. (A) MDCK cells were starved overnight in a 0% FBS medium, treated with HGF 100 ng/ml for 15 minutes in presence or absence of 1μM R59949 and lysed. GTP-bound active Rac was purified in each sample by pull-down with GST-fused PAK CD domain. Densitometric analysis was performed and the ratio of Rac-GTP *vs* total Rac was calculated for each sample. Activation is expressed as percentage of activation *vs* HGF-treated cells. Means of three experiments, with standard errors, are shown. * = $p<0.05$. (B) PINCOS- and PINCOS/Dgkα-DN-infected MDCK cells were starved overnight in a 0% FBS medium, treated with HGF 100 ng/ml for 15 minutes and pull-down assays were performed as described in (A).

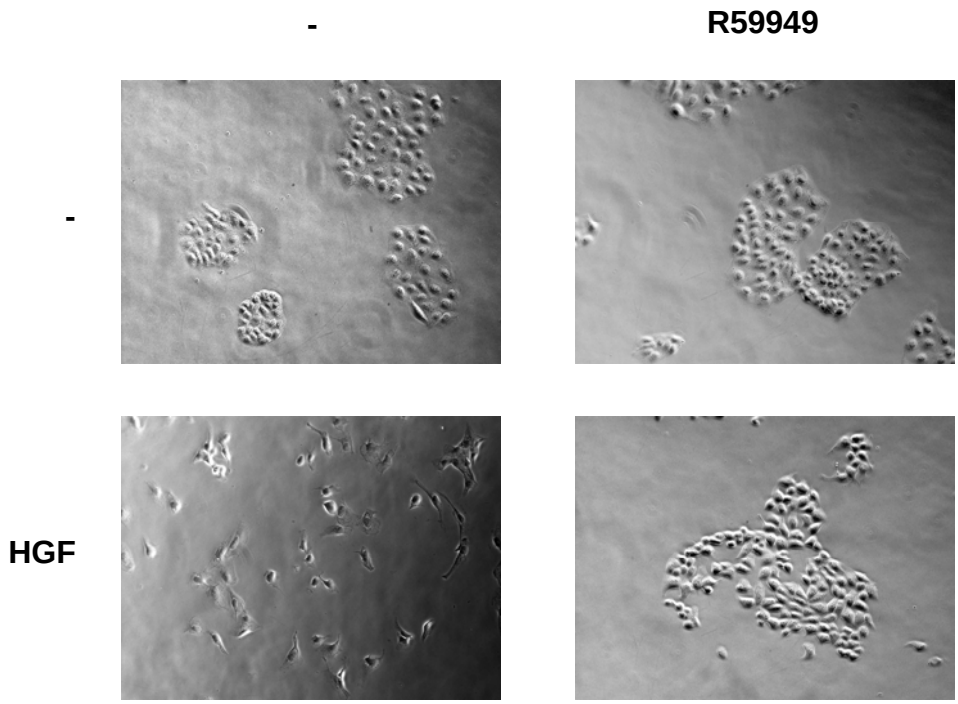
**Figure 7.** Dgkα is required for HGF-induced Rac localization to the plasma membrane. (A) MDCK cells colonies were starved overnight in a 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for Rac (green, panels a,d,g,j) and actin (red, panels b,e,h,k). Representative pictures are shown. The arrow indicates Rac staining at the outer plasma membrane of an edge-of-colony cell. Scale bar = 16 μm. (B) Confocal acquired images were observed and cells at the edge of colonies were scored as featuring Rac at the outer plasma membrane. Percentage of cells with Rac at the outer plasma membrane were calculated. Means of three experiments with standard errors are shown. * = $p<0.05$. (C) PINCOS- or PINCOS/Dgkα-DN-infected MDCK cells were starved overnight in a 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for Rac (red, panels a,d,g,j). Panels b,e,h,k show infected cells (green). The thick arrow indicates Rac at the plasma membrane in a PINCOS-infected cell; slim arrows indicate Rac at the plasma membrane of uninfected cells. Representative pictures are shown. Scale bar = 20 μm. (D) Confocal acquired images were scored for Rac staining at the outer plasma membrane in cells at the edge of colonies. Means of three experiments with standard errors are presented. ** = $p<0.005$.

# Figure 1

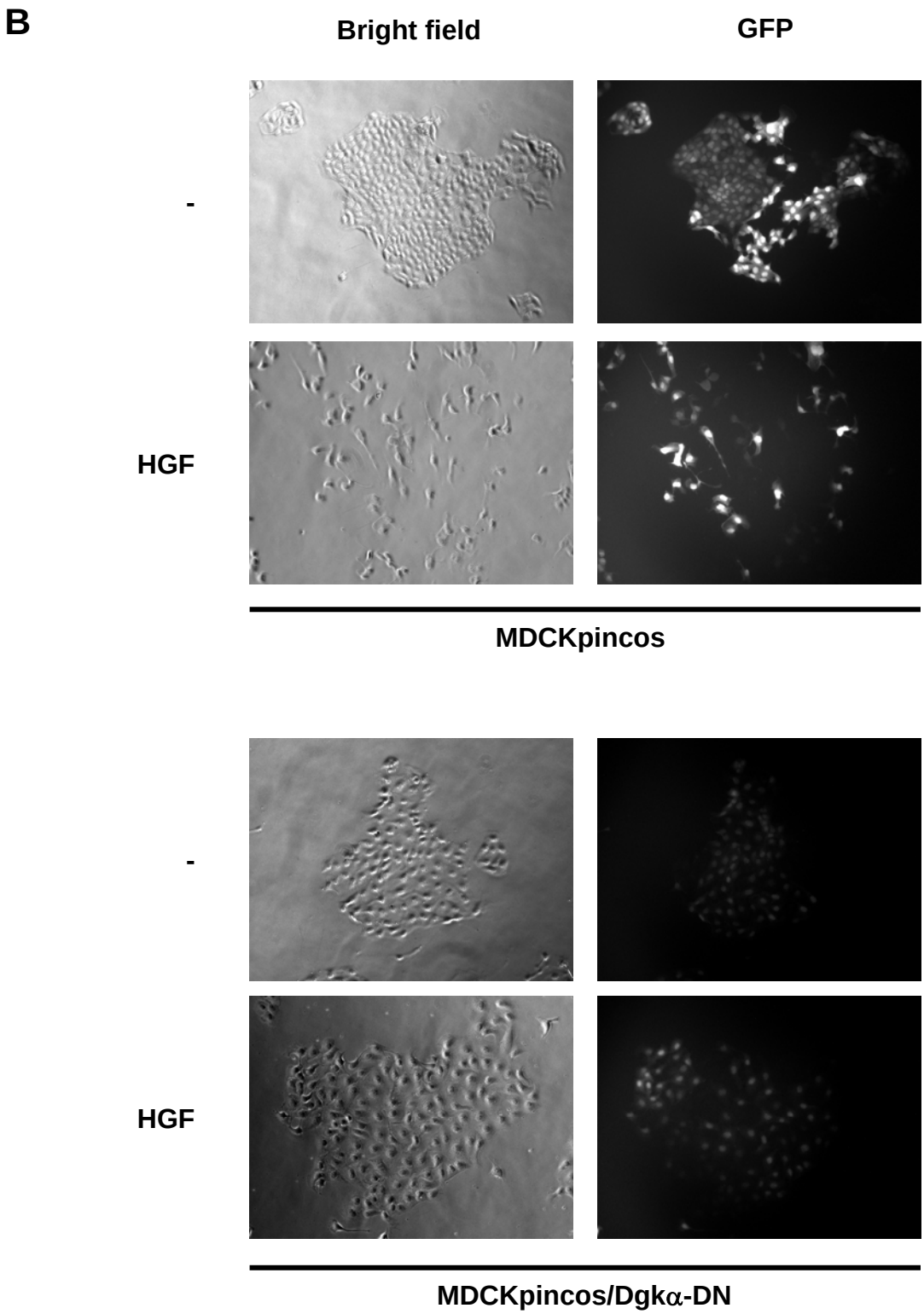

Transfection
empty vector
Myc-Dgkα
Temperature (°C)
40.5
40.5
35
30'
1h
4h
PA
IP anti Myc, Dgk assay
Myc-Dgkα
IP anti Myc, IB anti Myc

# Figure 2

**A**

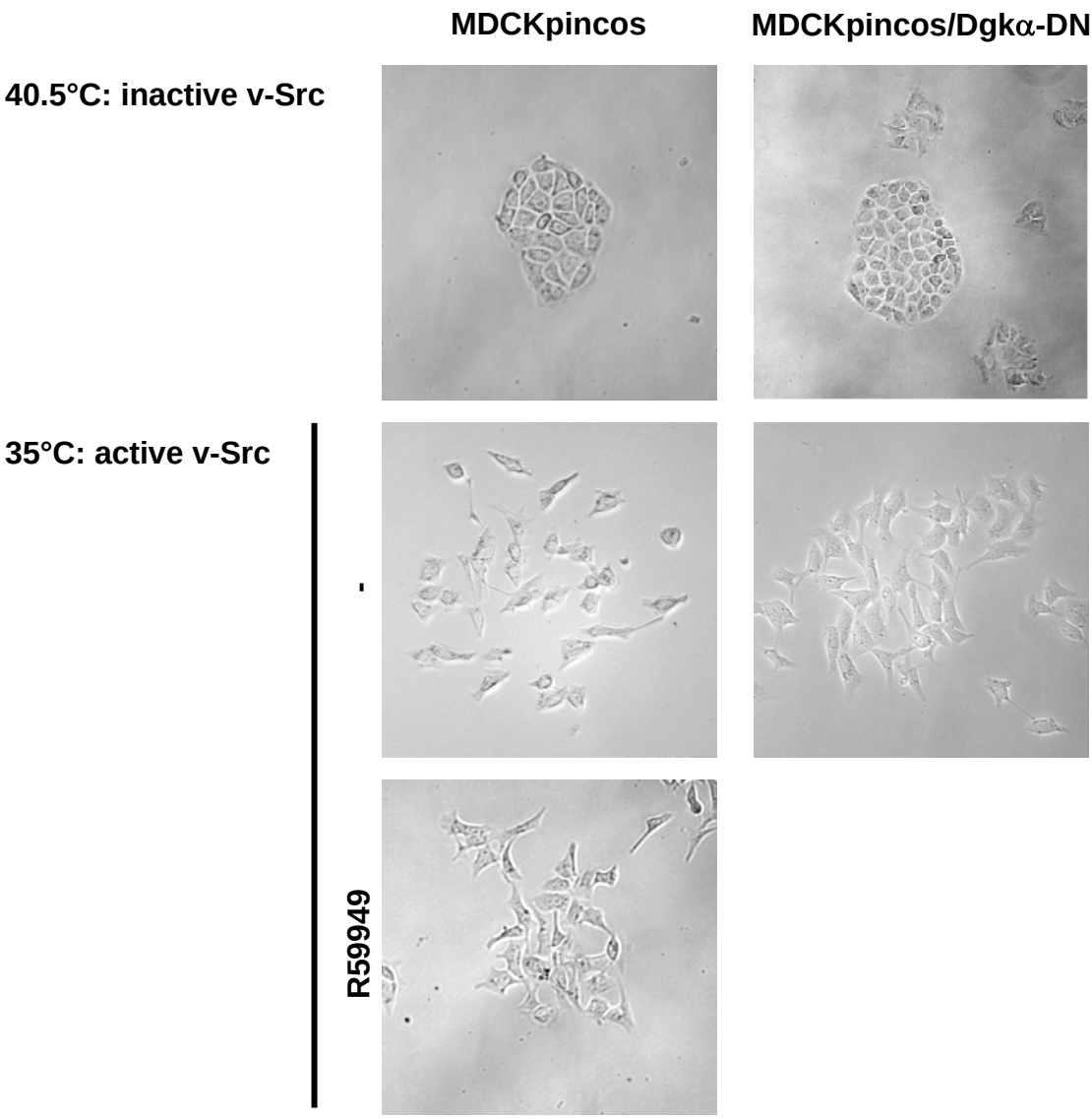


# (Figure 2)



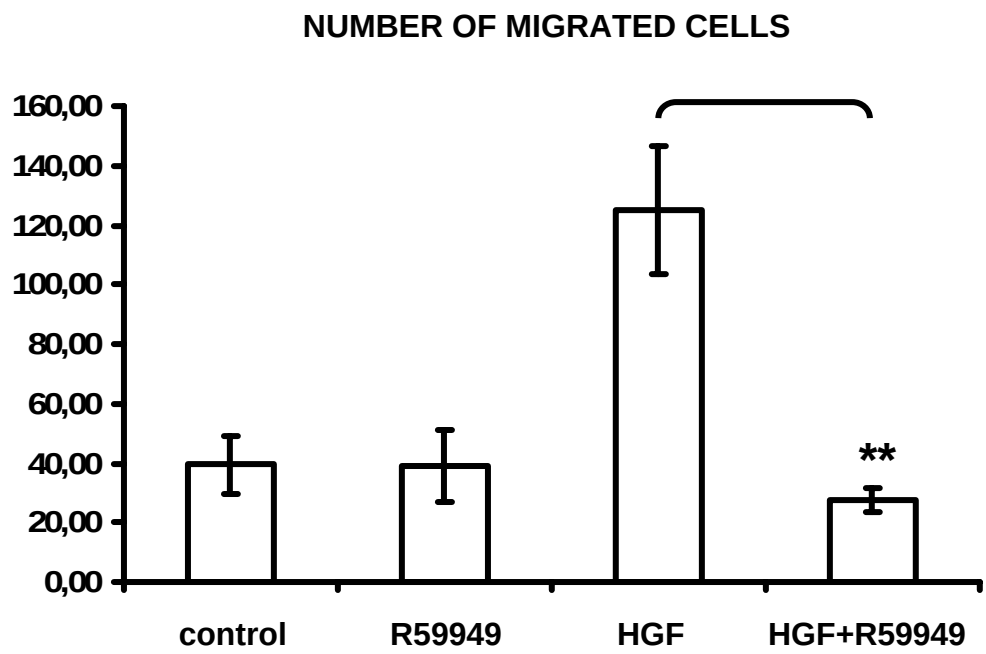
# (Figure 2)

**C**

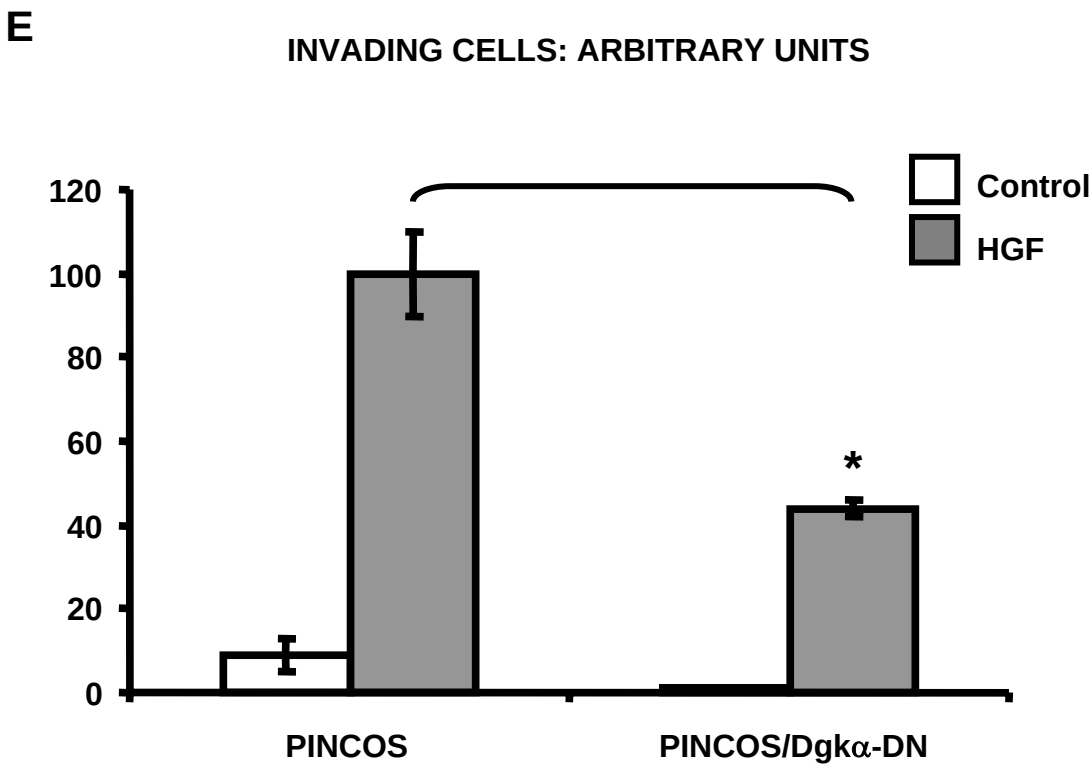

MDCKpincos
MDCKpincos/Dgkα-DN
40.5°C: inactive v-Src
35°C: active v-Src
-
R59949

**(Figure 2)**

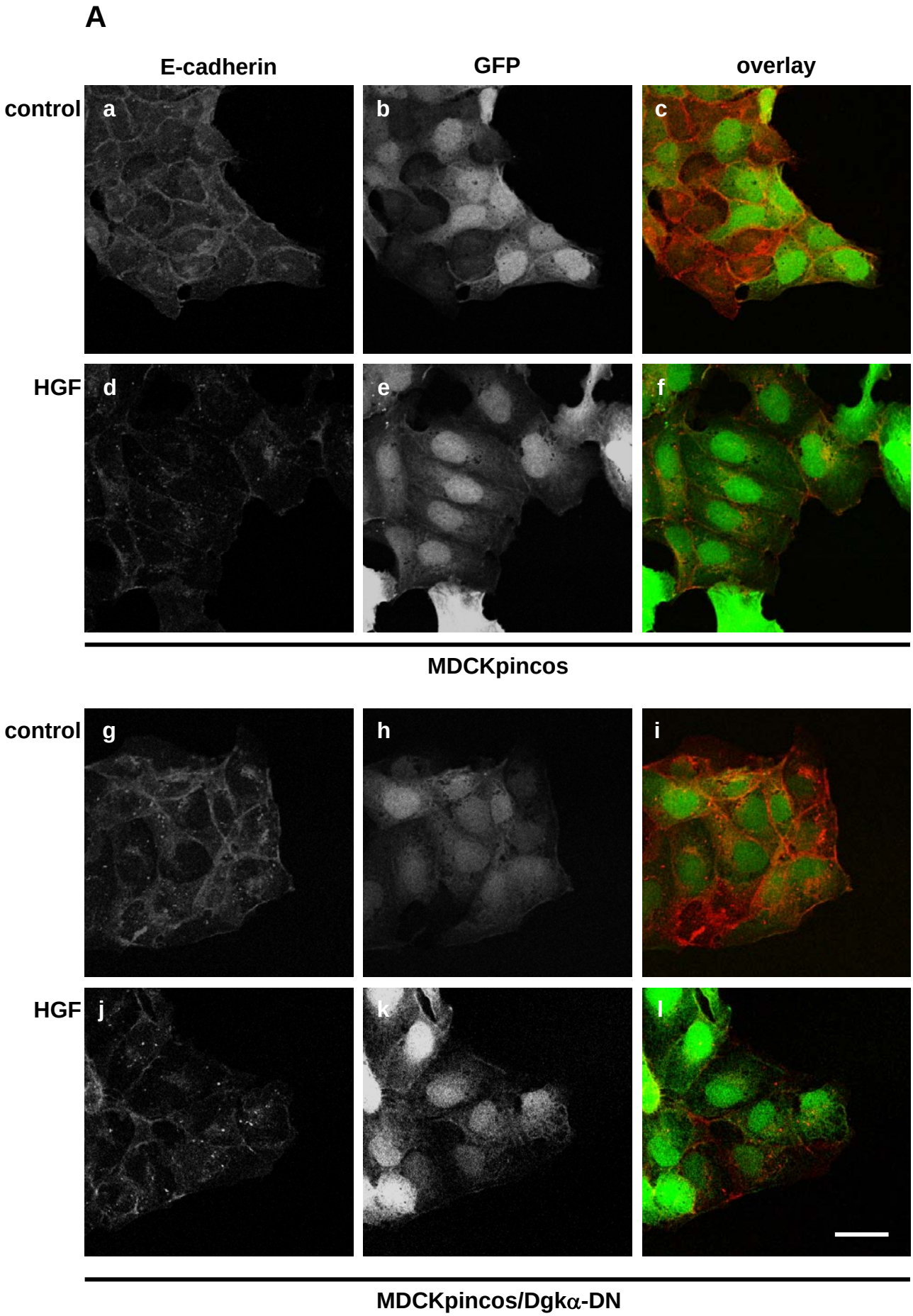
**D**


NUMBER OF MIGRATED CELLS
160,00
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
**
control
R59949
HGF
HGF+R59949

# (Figure 2)

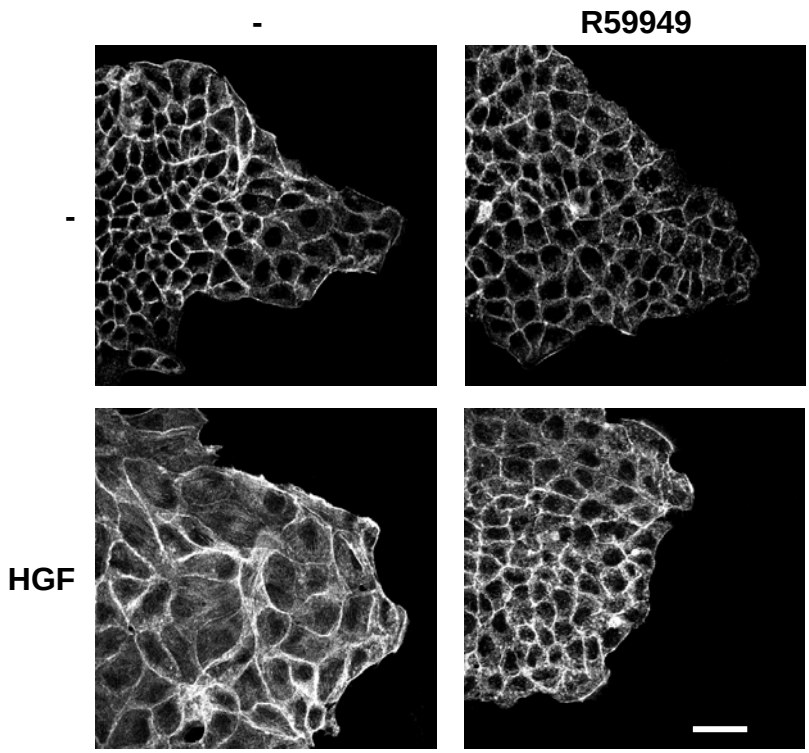

E
INVADING CELLS: ARBITRARY UNITS
120
100
80
60
40
20
0
Control
HGF
*
PINCOS
PINCOS/Dgkα-DN

# Figure 3


A
E-cadherin
GFP
overlay
control
HGF
a
b
c
d
e
f
MDCKpincos
control
HGF
g
h
i
j
k
l
MDCKpincos/Dgkα-DN

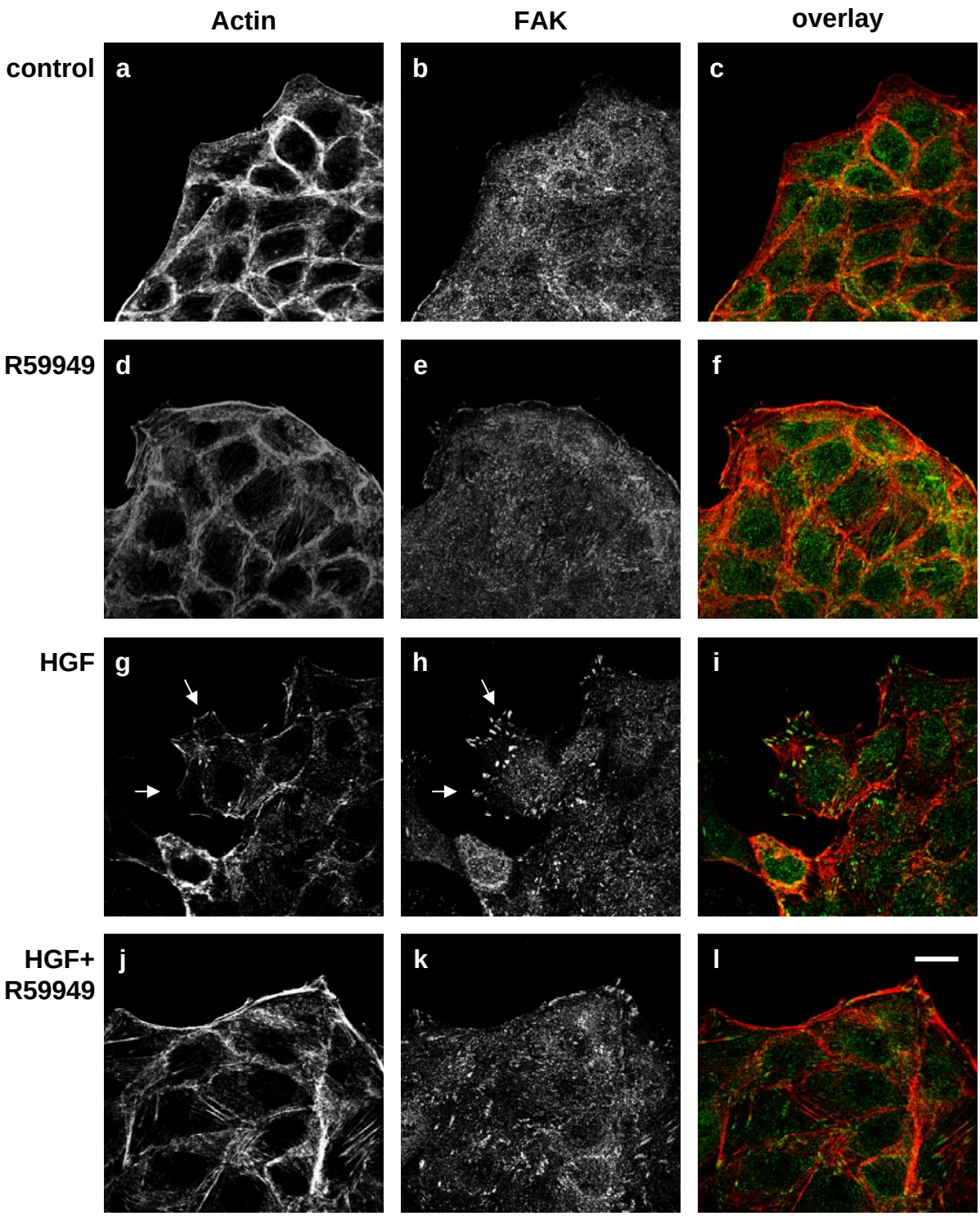
# (Figure 3)

**B**



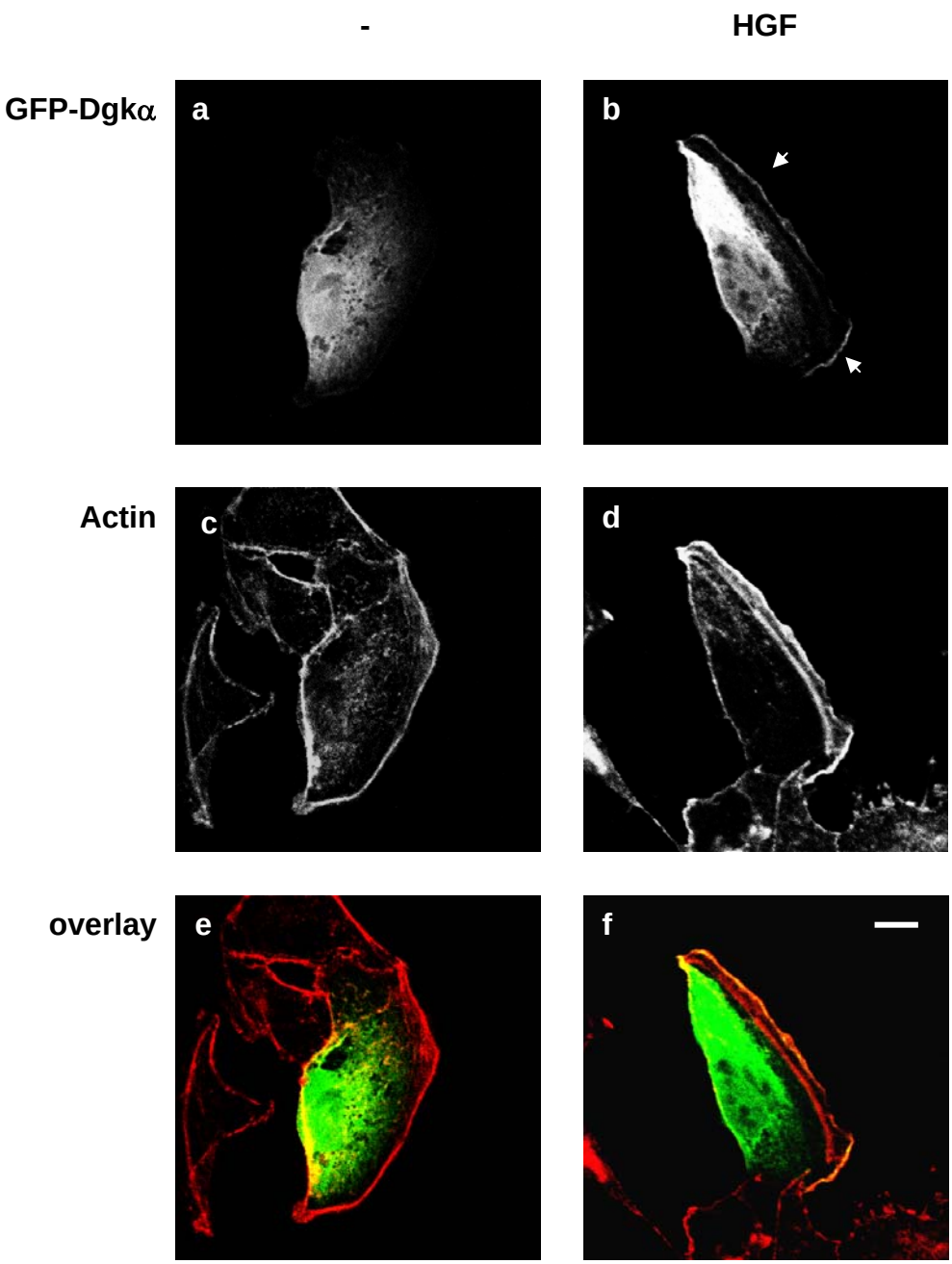
C

(Figure 3)


Actin
FAK
overlay
control
R59949
HGF
HGF+
R59949
a
b
c
d
e
f
g
h
i
j
k
l

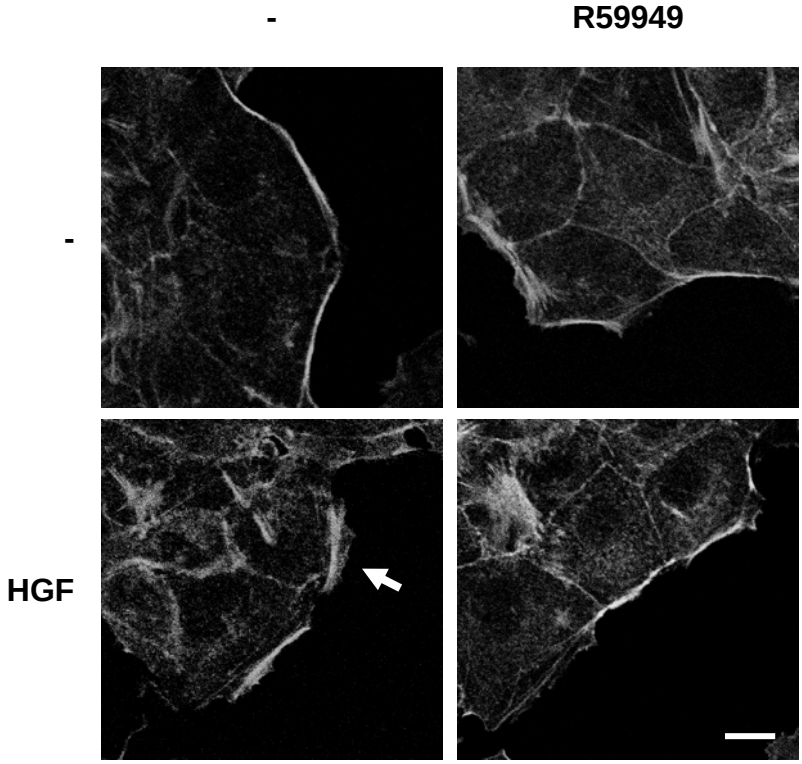
# Figure 4

**A**

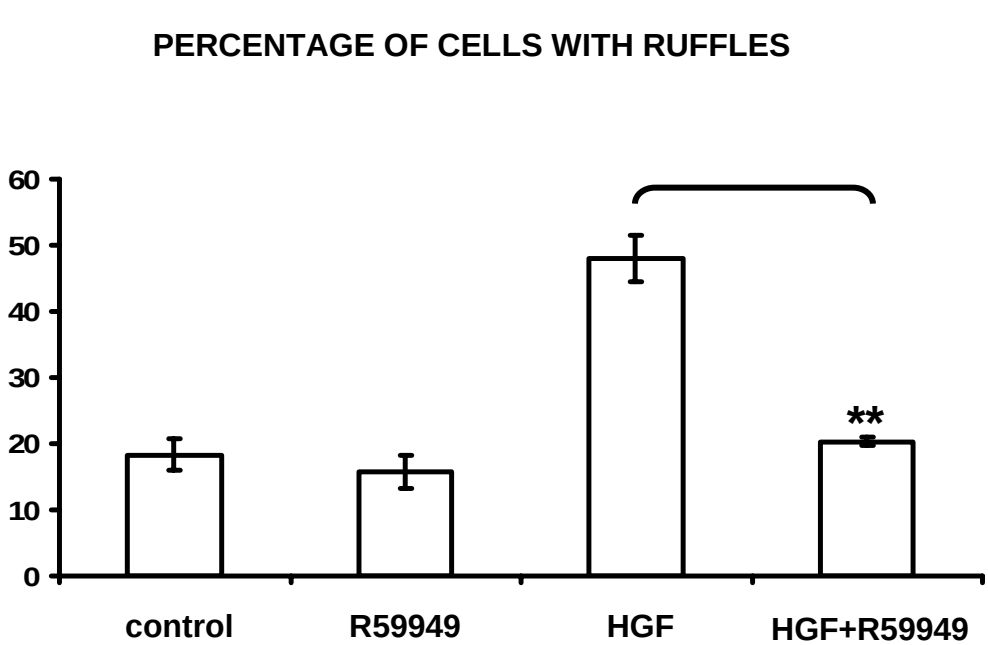


# (Figure 4)

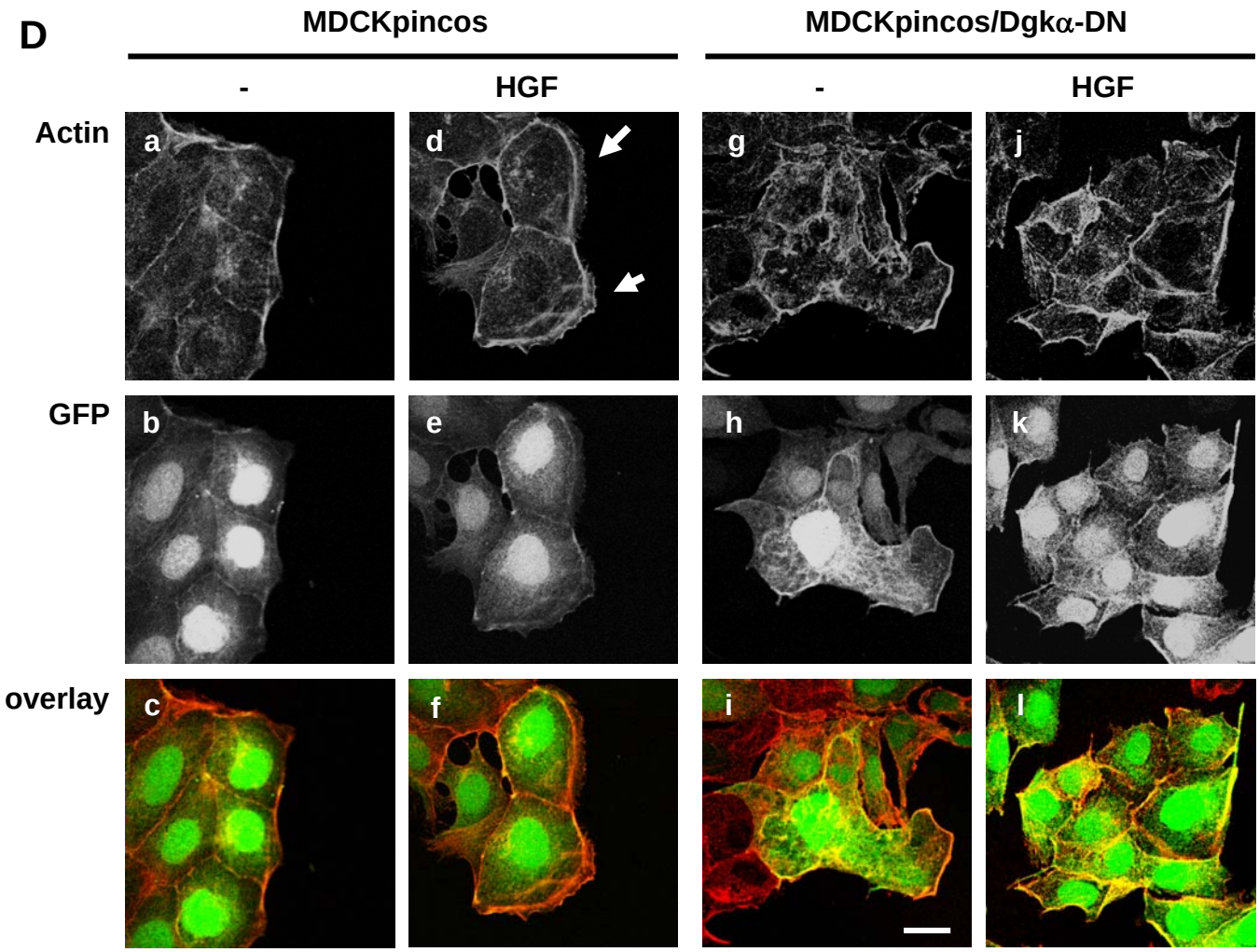
**B**

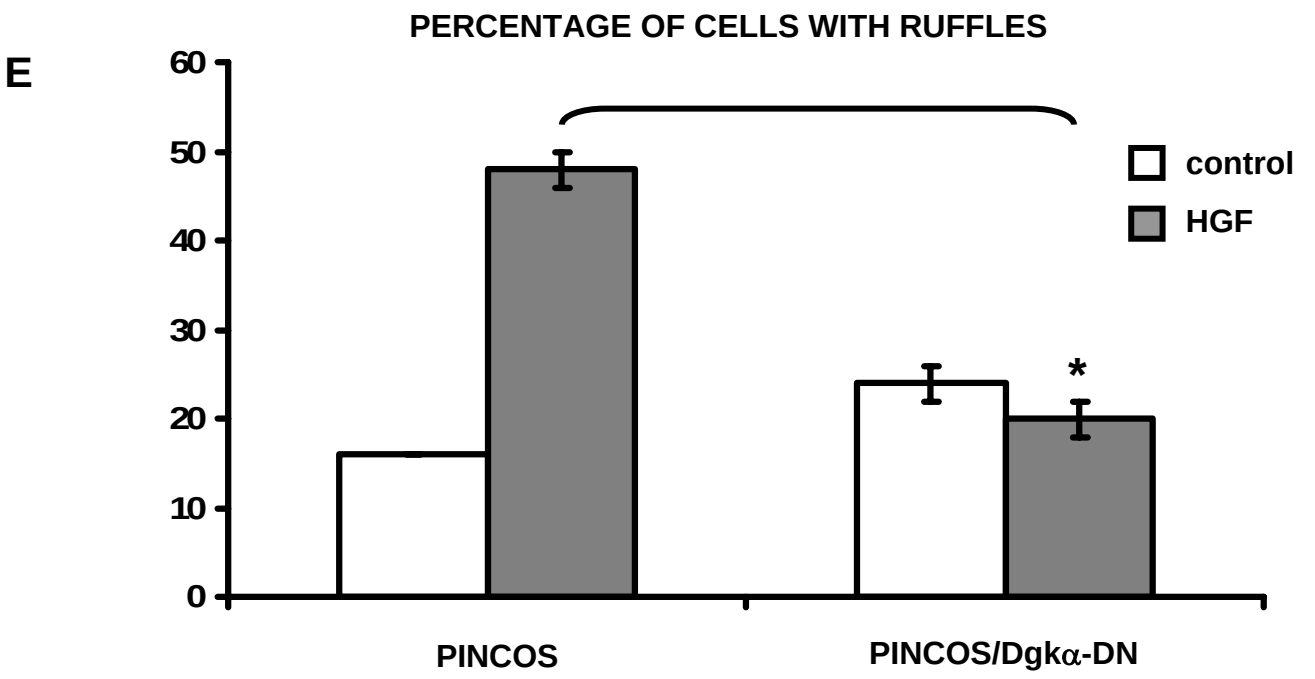

-
R59949
-
HGF

**C**

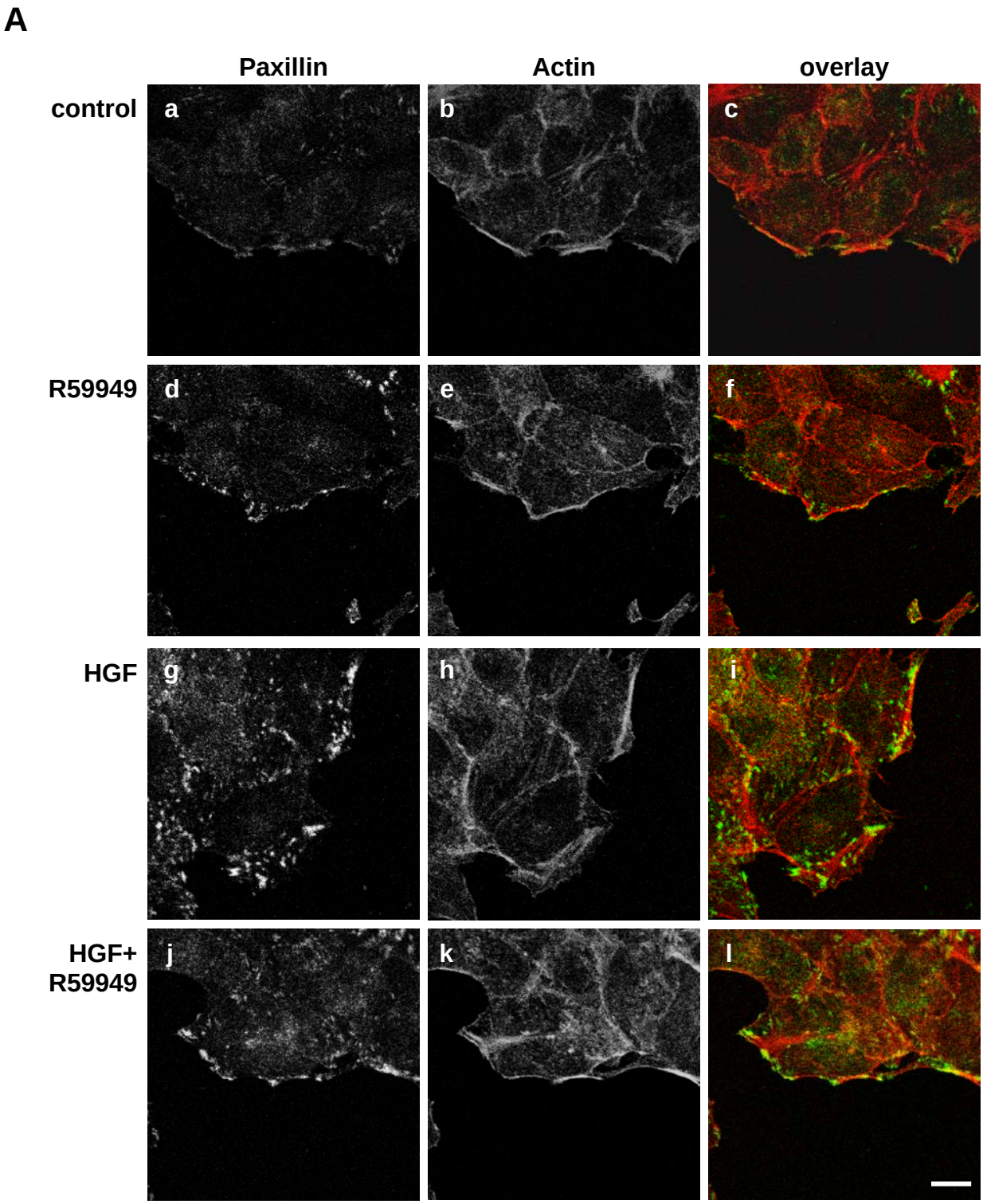

PERCENTAGE OF CELLS WITH RUFFLES
60
50
40
30
20
10
0
**
control
R59949
HGF
HGF+R59949

# (Figure 4)


D
MDCKpincos
MDCKpincos/Dgkα-DN
-
HGF
-
HGF
Actin
GFP
overlay
a
b
c
d
e
f
g
h
i
j
k
l

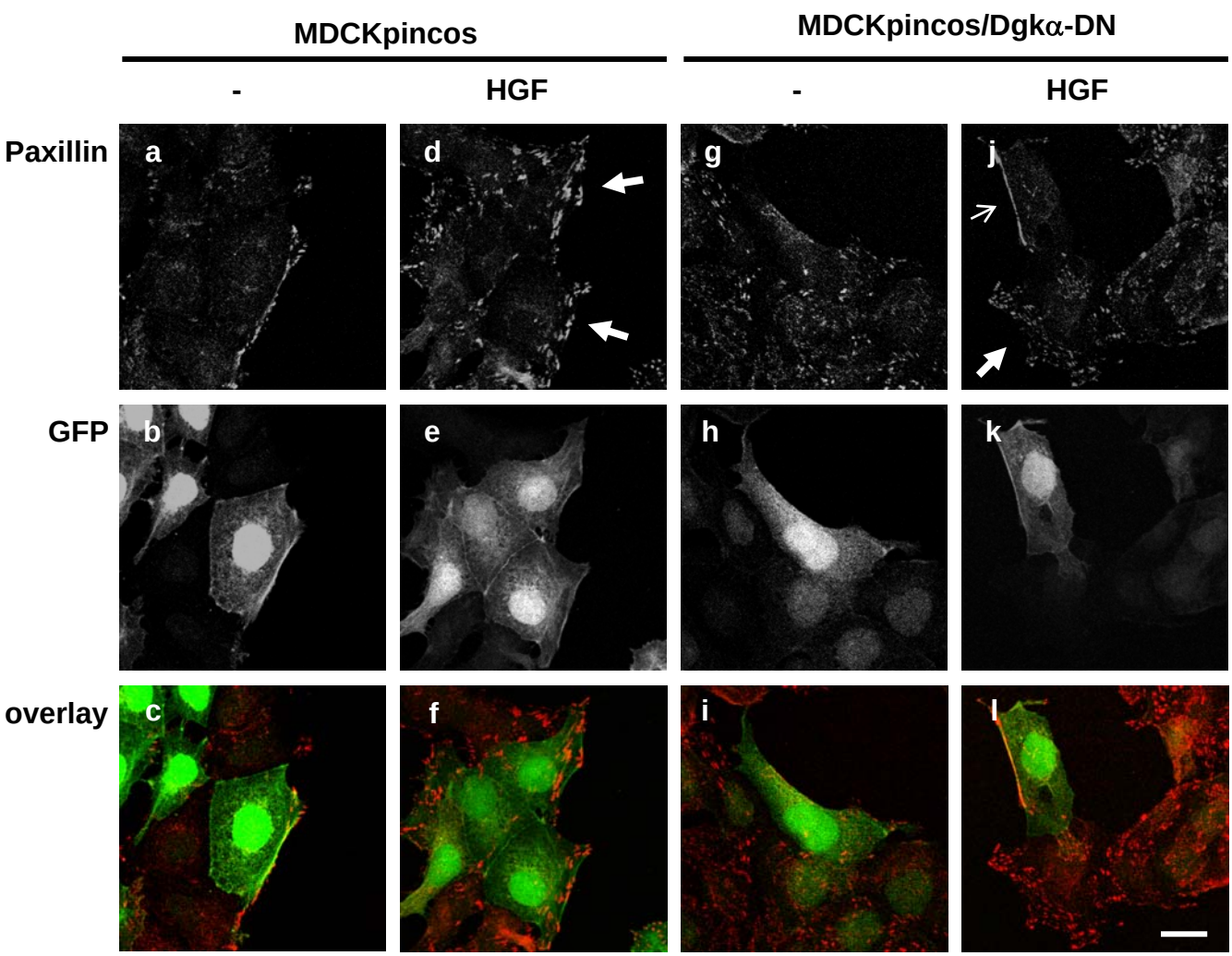

E
PERCENTAGE OF CELLS WITH RUFFLES
60
50
40
30
20
10
0
control
HGF
*
PINCOS
PINCOS/Dgkα-DN

# Figure 5


A
Paxillin
Actin
overlay
control
R59949
HGF
HGF+
R59949
a
b
c
d
e
f
g
h
i
j
k
l

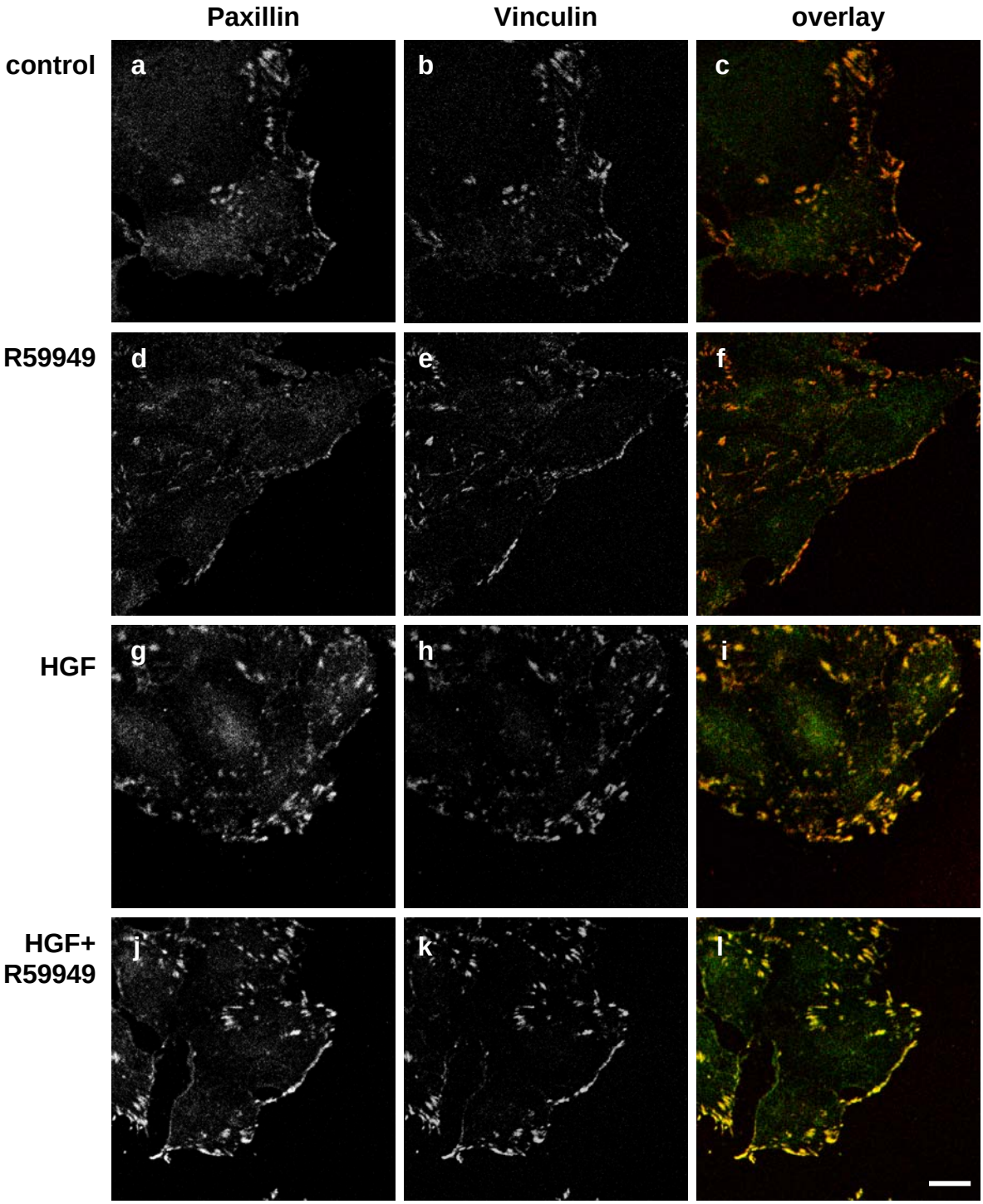
# (Figure 5)

**B**


MDCKpincos
MDCKpincos/Dgkα-DN
-
HGF
-
HGF
Paxillin
GFP
overlay
a
b
c
d
e
f
g
h
i
j
k
l

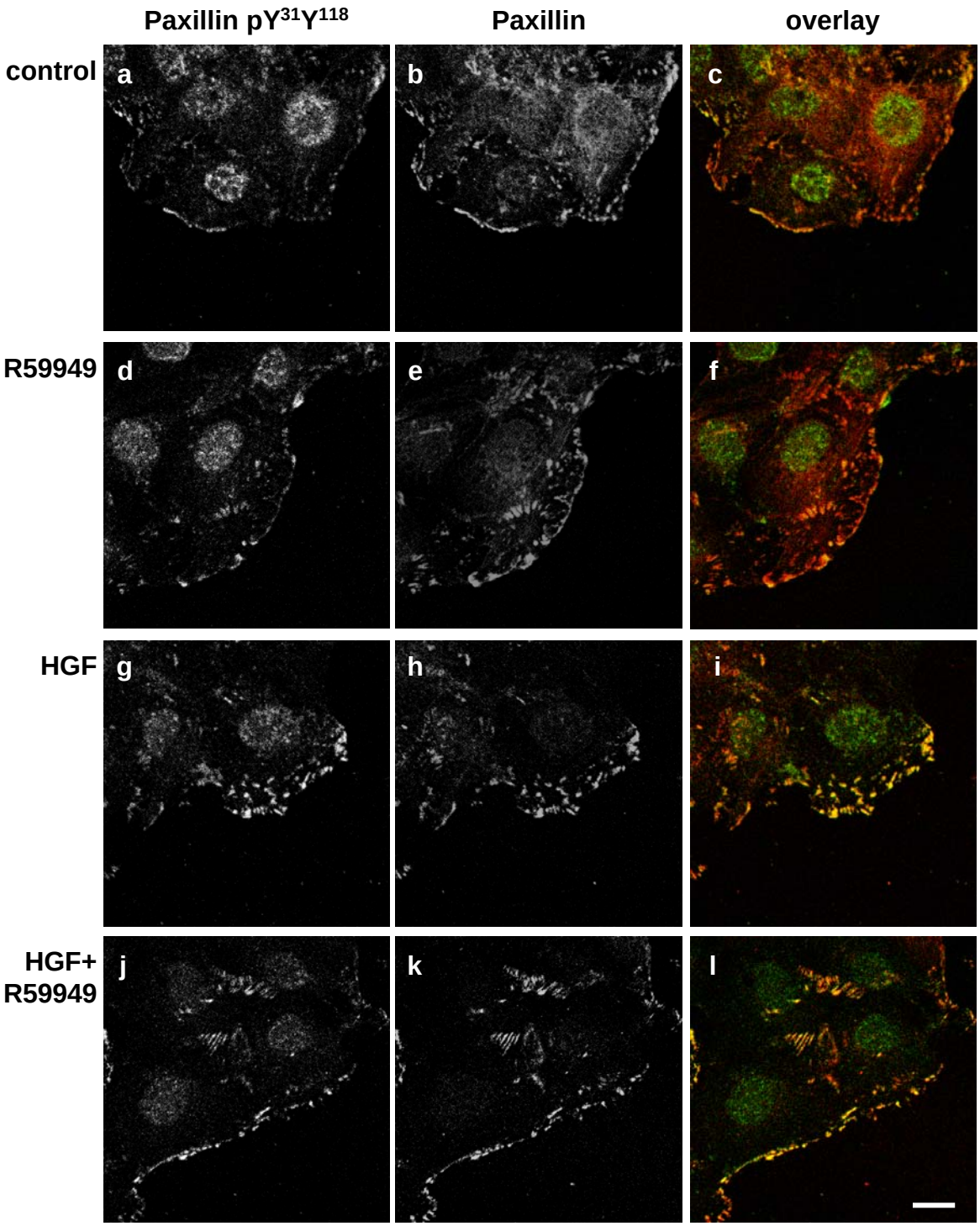
# (Figure 5)

**C**


Paxillin
Vinculin
overlay
control
R59949
HGF
HGF+
R59949
a
b
c
d
e
f
g
h
i
j
k
l

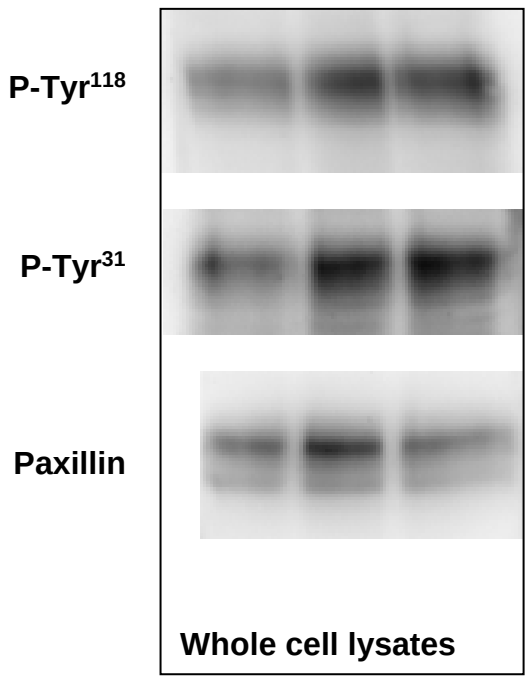
# (Figure 5)

**D**


Paxillin pY31Y118
Paxillin
overlay
control
R59949
HGF
HGF+ R59949
a
b
c
d
e
f
g
h
i
j
k
l

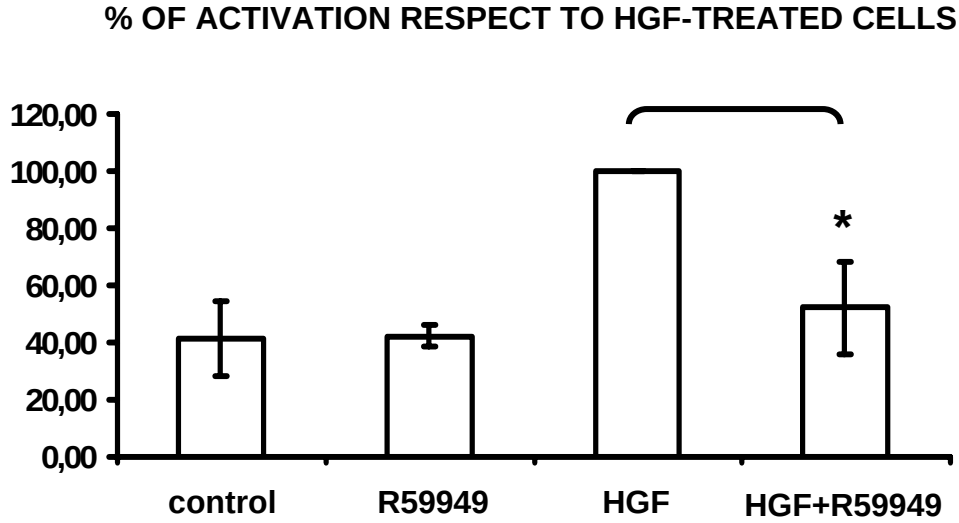
# (Figure 5)

**E**

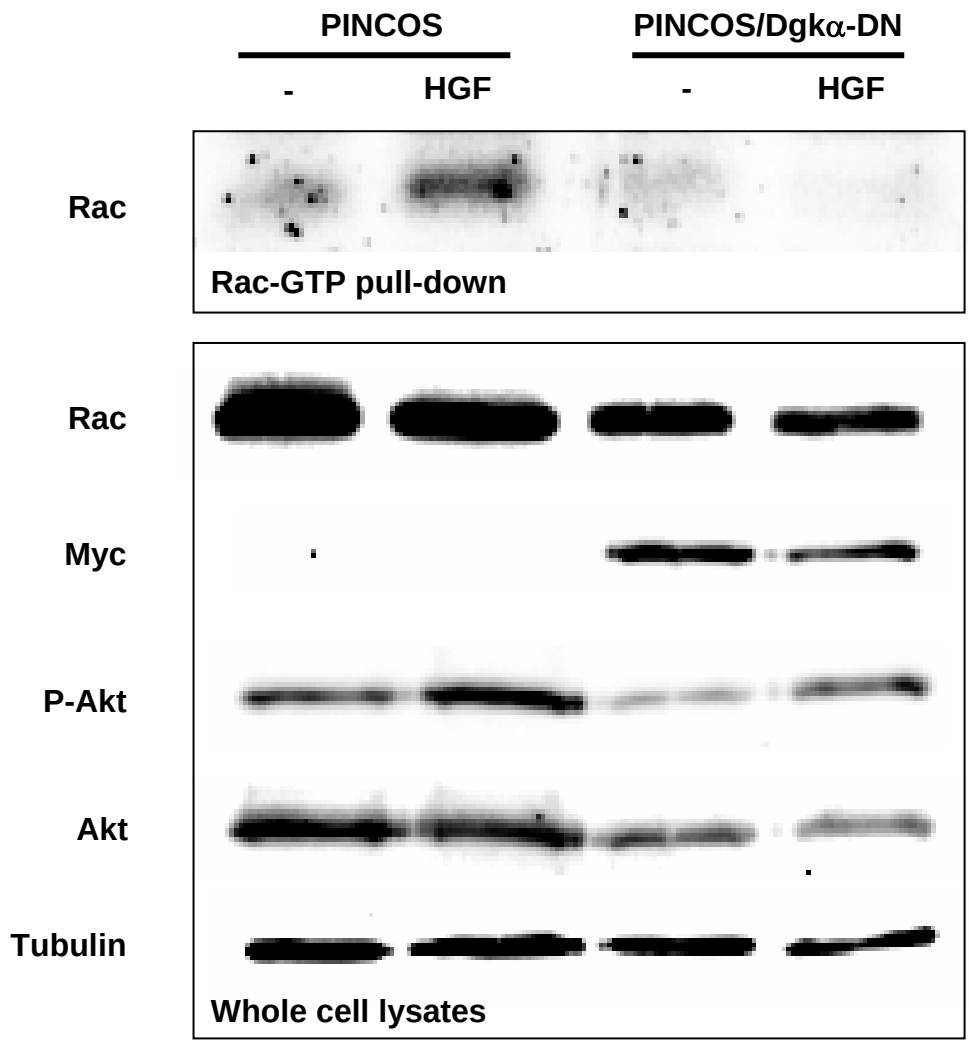


# Figure 6

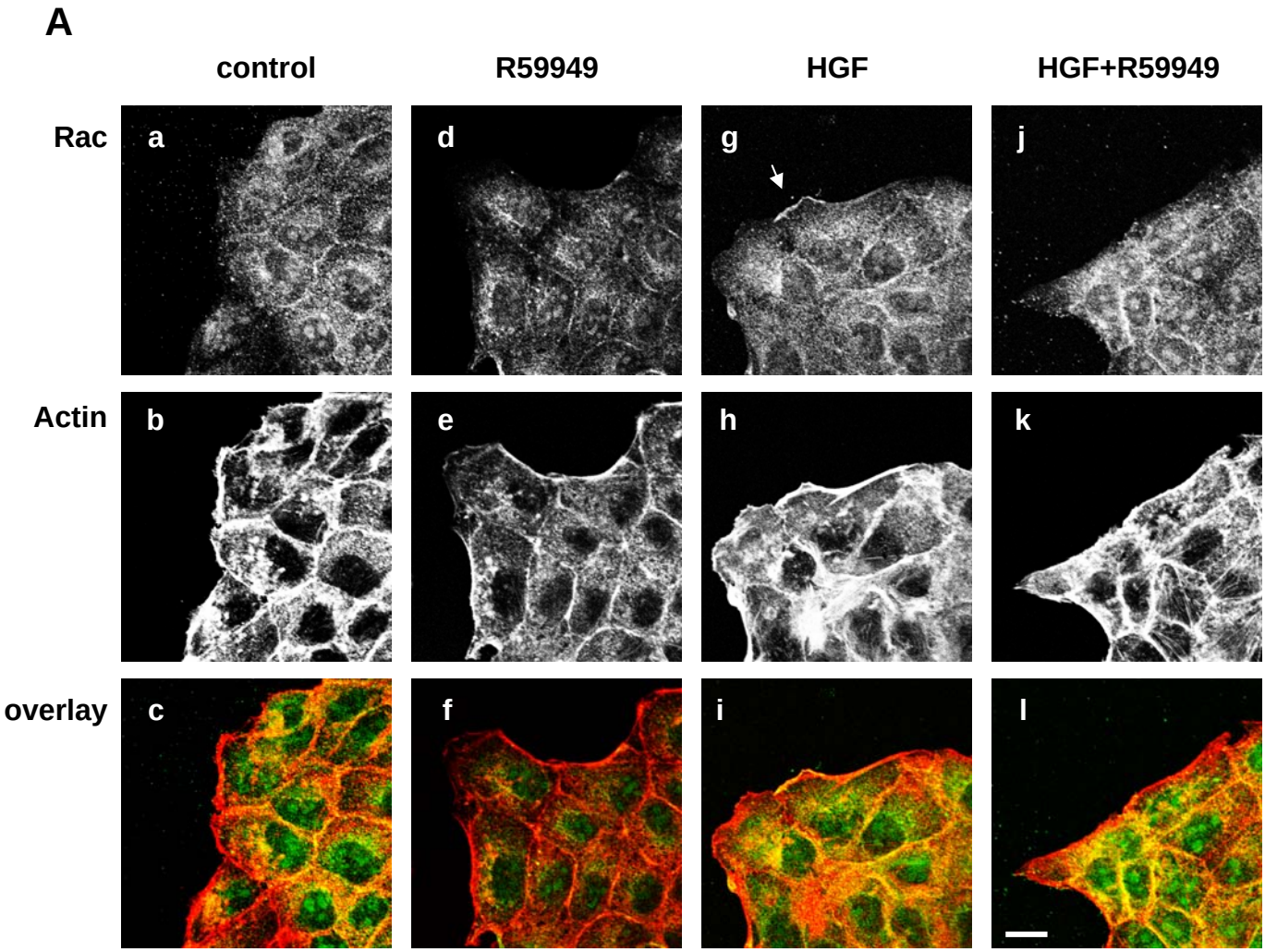
**A**

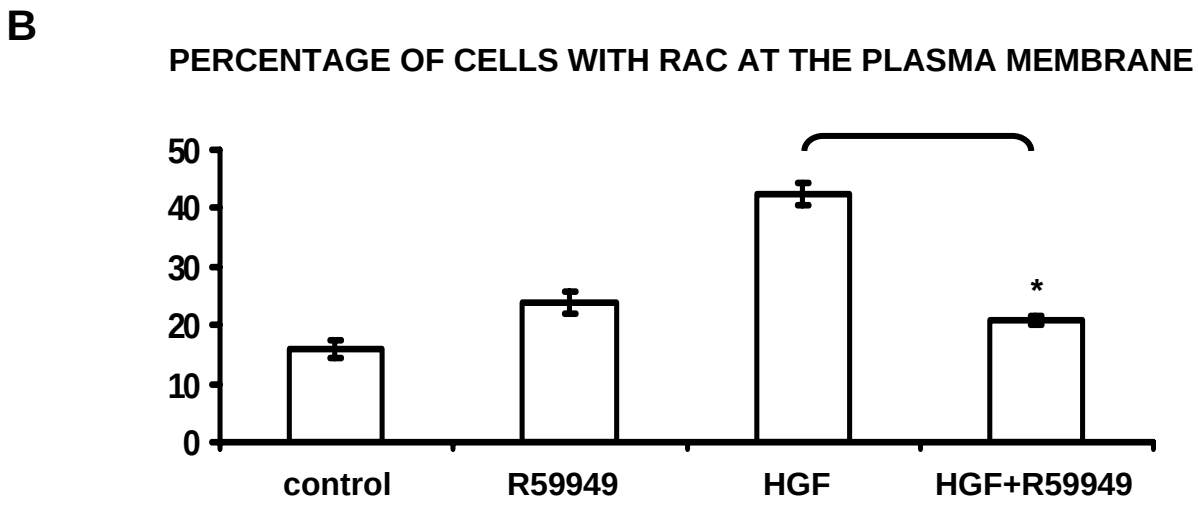

% OF ACTIVATION RESPECT TO HGF-TREATED CELLS
120,00
100,00
80,00
60,00
40,00
20,00
0,00
*
control
R59949
HGF
HGF+R59949

**B**


PINCOS
PINCOS/Dgkα-DN
-
HGF
-
HGF
Rac
Rac-GTP pull-down
Rac
Myc
P-Akt
Akt
Tubulin
Whole cell lysates

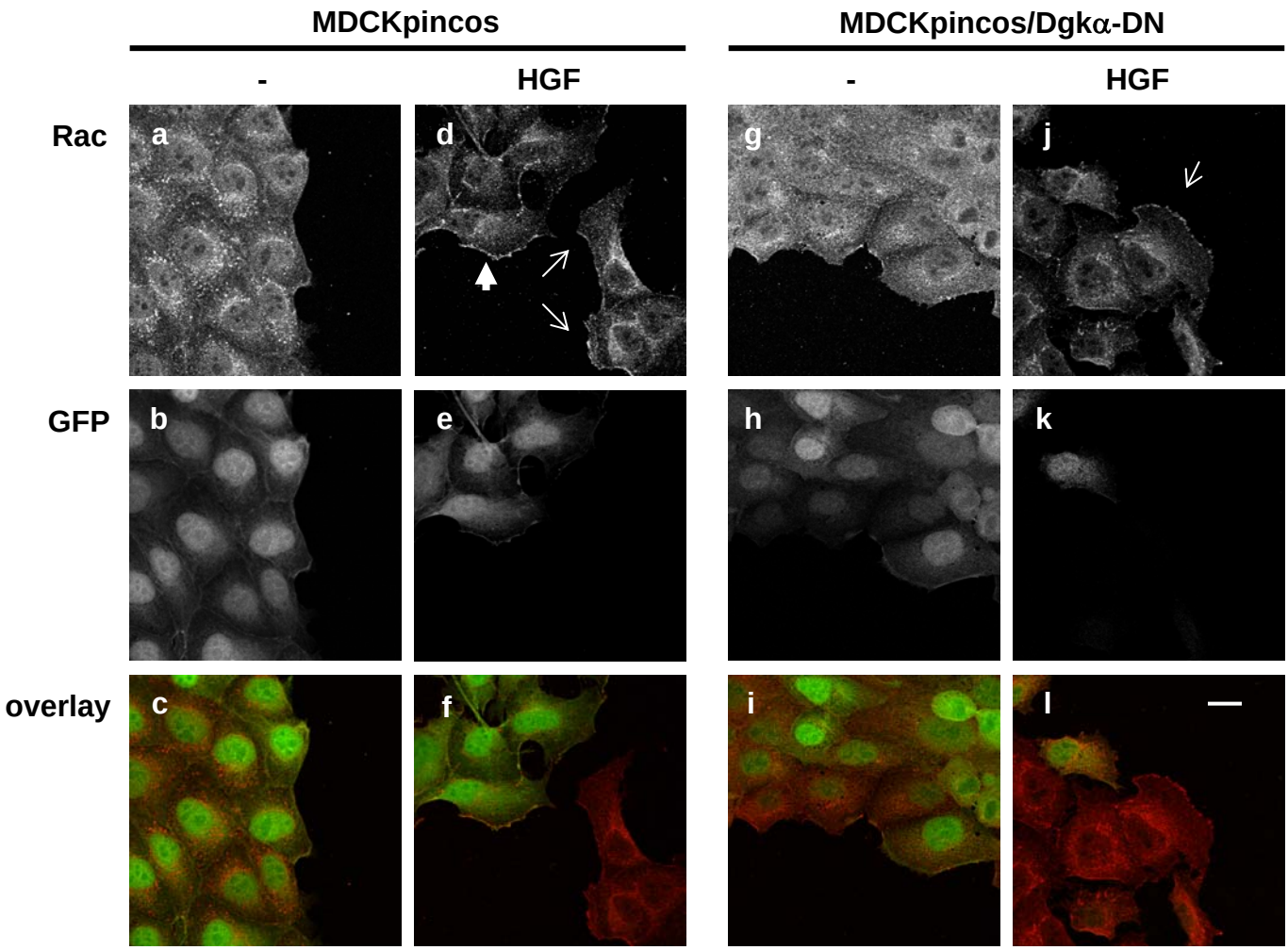
# Figure 7


A
control
R59949
HGF
HGF+R59949
Rac
Actin
overlay
a
b
c
d
e
f
g
h
i
j
k
l

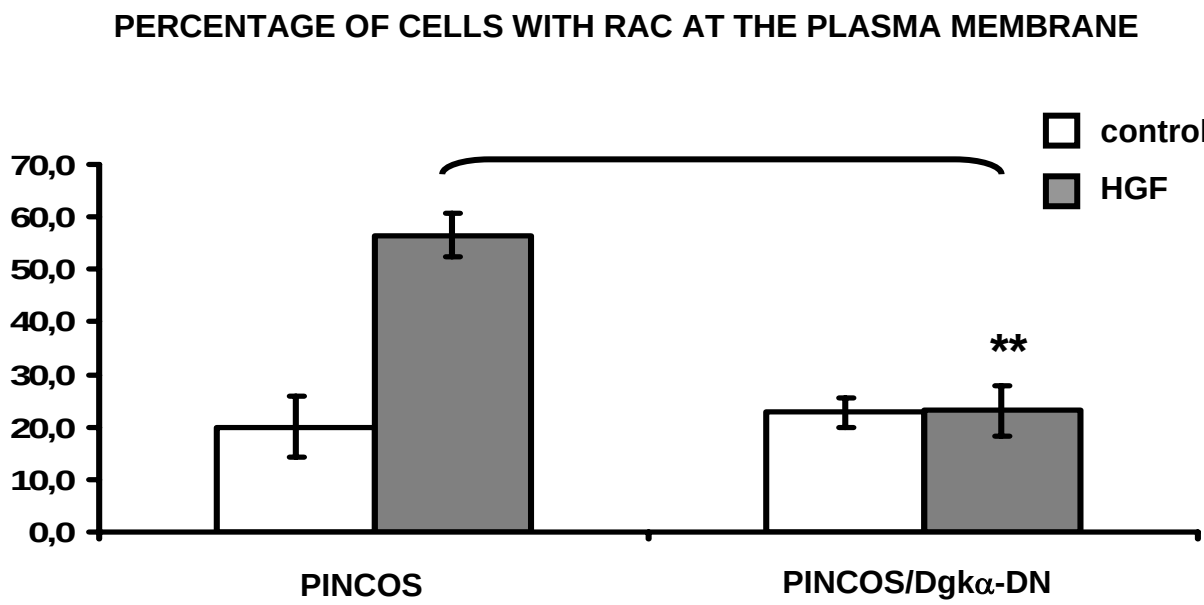

B
PERCENTAGE OF CELLS WITH RAC AT THE PLASMA MEMBRANE
50
40
30
20
10
0
*
control
R59949
HGF
HGF+R59949

# (Figure 7)

**C**


MDCKpincos
MDCKpincos/Dgkα-DN
-
HGF
-
HGF
Rac
GFP
overlay
a
b
c
d
e
f
g
h
i
j
k
l

**D**


PERCENTAGE OF CELLS WITH RAC AT THE PLASMA MEMBRANE
control
HGF
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
**
PINCOS
PINCOS/Dgkα-DN