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Relazione scientifica dell'attività svolta (in inglese).

(A) Diacylglycerol kinase-α mediates HGF-induced epithelial cell scatter by regulating membrane ruffling.

Project aim/objectives.

The aim of this project is the study of the role of diacylglycerol kinase α (Dgk α) in HGF-induced cell migration; in particular I focalized on the early events leading to epithelial cell migration, such as membrane ruffle formation and focal adhesion organization.

Background.

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) (Thiery, 2002; Thiery *et al.*, 2006). *In vitro*, the scattering of epithelial cells, *i.e.* the dispersal of colonies due to loss of intercellular adhesions and acquisition of cell motility, is triggered by growth factor stimulation and by oncogene activation, recapitulating the early phases of EMT (Avizienyte and Frame, 2005).

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 SOS, RhoGDI, Ras- and Rho-GAPs, and

signalling lipid metabolizing enzymes, such as PI(4)P 5-kinase and PLC- γ (Topham, 2006; Zhao *et al.*, 2007). 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.

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 phosphorylation of Dgkα on Tyr^{335} by Src itself (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2007). The specific signalling pathways regulated by activation of Dgkα still await elucidation. We have also shown that Dgkα mediates HGF-induced cell scatter and migration of MDCK cells. Here, I show that Dgkα activation is required in those mechanisms leading to F-actin cytoskeleton and focal adhesion remodelling and formation of membrane ruffles.

Experimental plan and methods.

Cell culture. MDCK (Madin-Darby canine kidney cells) are a kind gift of W. Birchmeier (Berlin). Cells were cultured in high glucose DMEM GlutaMAX™ medium (Gibco), supplemented with 10% fetal bovine serum (Gibco) and antibiotic-antimicotic solution (Sigma), in humidified atmosphere with 5% CO_2 at 37°C.

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 as R59949. Anti-Myc was from Upstate, anti-Paxillin from BD Transduction Laboratories, anti-Paxillin $pTyr^{31}$ and $pTyr^{118}$ from Biosource, anti-α-Tubulin from Sigma, anti-Vinculin from Novus Biological, Alexa Flour 546/633 Phalloidin from Molecular Probes. Anti-Dgkα was kindly provided by W.J. van Blitterswijk (the Netherlands Cancer Institute, Amsterdam). 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 has been previously described (Cutrupi *et al.*, 2000). GFP-Dgkα-WT (wild type) was obtained by cloning Dgkα in pcDNADEST53 (Invitrogen) using Gateway kit (Invitrogen) according to manufacturer's instructions. Briefly, Dgkα cDNA was inserted in pDONOR 2.11 vector by PCR and BP recombination. LR recombination was performed to transfer Dgkα in pcDNA-DEST53 for N-terminal GFP fusion. $G_{434}D$ point mutation on Dgkα to obtain the kinase-defective dominant negative mutant (GFP-Dgkα-DN) was performed using QuikChange Site-Directed Mutagenesis Kit 22 (Stratagene) as previously described (Cutrupi *et al.*, 2000). PINCOS retroviral vector and PINCOS/Dgkα-DN, expressing both GFP and the inserted gene, have already been

described (Cutrupi *et al.*, 2000). Transient transfections were performed using Lipofectamine2000 Reagent (Invitrogen) according to the manufacturer's instructions.

MDCK cells stably expressing PINCOS/empty vector or PINCOS/Dgkα-DN were obtained by infection. Briefly, GP2-293 packaging cell line (Clontech, kindly provided by R. Piva, University of Torino) 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. The next day the medium was changed to normal growth medium. Forty-eight hours after infection, the retroviral supernatant was collected, the debris removed by centrifugation at 1500g, and the supernatant was filtered 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 adding 2 ml of retroviral supernatant and 1 ml of growth medium. The day after the first infection cells were re-infected as previously described. 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 GFP expressing cells.

The murine Dgkα, resistant to canine Dgkα siRNAs, was cloned in the lentiviral vector pLenti4V5 (Invitrogen). Lentiviruses were produced following the manufacturer's instructions and used to infect MDCK cells, which were then selected in Zeocin-containing medium to obtain a stably-expressing cell line.

RNA interference. siRNAs against canine Dgkα were chemically synthesized as double-strand RNA (Ambion). Sequences were as follows: C1 sense GCUCAGAAGUGGACAGGAUtt antisense AUUCUGUCCACUUCUGAGCtg; C2 sense CCCAGACAUCCUGAAAACCtt antisense GGUUUUCAGGAUGUCUGGGtc; C3 sense CCUUCCACACCACAAAAACtt antisense GUUUUUGUGGUGUGGAAGGtg. A GAPDH scramble siRNA (Ambion) was used as negative control. The BLOCK-iT^{TM} Fluorescent Oligo (Invitrogen) is a fluorescein-labelled dsRNA oligomer and was used to obtain indication of the transfection efficiency with siRNAs.

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). After stimulation, cells were washed twice in PBS and fixed by incubation with PBS 3% paraformaldheyde-4% sucrose. Cells were then permeabilized in 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), washed with PBS containing 0.2% BSA and incubated for 15 minutes with PBS containing 2% BSA. 10 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-/TRITC-conjugated secondary antibodies and/or Alexa Fluor 546/633 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 pH 7.4) and let polymerize. Confocal images were acquired with the Leica confocal microscopy TSP2 and LCS Leica confocal 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 direct western blotting.

Statistical analysis. At least triplicates were analysed when quantification was performed. Couples of conditions were compared using Student t-test. Histograms represent means ± standard errors.

Results.

Dgkα is required for HGF-induced membrane ruffle formation.

We have previously shown that Dgkα is required for cell scatter, invasion and chemotaxisis induced by hours of HGF stimulation, without affecting the loss of E-cadherin-mediated intercellular adhesions.

Upon few minutes of HGF stimulation, MDCK 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 ascertained Dgkα localization in resting or HGF-treated MDCK cells 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 1A). 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 1B, arrows). The percentage of cells featuring membrane ruffles raises from less than 20% in control cells (vehicle- and R59949-treated cells) to about 50% in HGF-treated cells. In presence of 1μM R59949, the percentage of membrane ruffle displaying cells upon HGF stimulation is reduced to almost control value (Figure 1B). In order to further verify the specificity of

Dgkα requirement in HGF-induced cell scatter, the endogenous protein was down-regulated by transient transfection of specific siRNAs. Three siRNAs were designed (C1, C2, C3), transiently transfected in MDCK cells, and proved to be effective in knocking down canine Dgkα, as verified by western blot; negative control siRNA does not affect Dgkα expression (Figure 2A). Transfection of MDCK cells, in the same conditions, with BLOCK-iT Fluorescent Oligo demonstrates that the efficiency of siRNA internalization into MDCK cells is near to 100% (Figure 2A). Similarly to R59949 treatment, transient transfection of either C1, C2 or C3 siRNA impairs HGF-induced membrane ruffling and this inhibition is completely overridden by the expression of the Dgkα murine hortologue, which is not affected by any of the three siRNAs (Figure 2B). Consistently, HGF fails to induce membrane ruffles in cells expressing Dgkα-DN compared to cells expressing the vector alone (Figure 2C). In conclusion, these data demonstrate that the formation of membrane ruffles, occurring upon 15 minutes of HGF treatment, depends on stimulation of Dgkα activity.

Dgkα is required for the remodelling of Paxillin- and Vinculin-containing focal complexes.

Membrane ruffle 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 molecules, 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 3A and B, panel a). Upon 15 minutes of HGF stimulation, Paxillin condensates to the newly-formed focal complexes, in correspondence of membrane ruffles (Figure 3A, panel c and Figure 3B, panel b). Upon inhibition of Dgkα by either 1 μM R59949 (Figure 3A, panel d), or by expression of Dgkα-DN (Figure 3B, panel f), Paxillin accumulates along the outer plasma membrane instead of being recruited in the area of ruffling, while ruffle 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 in structures identifiable as focal complexes, we analyzed its co-localization with Vinculin, a resident protein whose function is to stabilize them (Ziegler *et al.*, 2006). In unstimulated cells, Vinculin and Paxillin co-localize at focal complexes along the outer plasma membrane of colony-edge cells and upon HGF stimulation they are both recruited to newly-formed focal complexes in the area of ruffling, in a manner fully dependent on Dgkα activity. In fact, inhibition of Dgkα, while impairing HGF-induced neoformation of ruffles and focal complexes at membrane ruffles, does not affect Vinculin and Paxillin co-localization (Figure 4A).

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 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 factor-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 Paxillin Tyr^{31} and Tyr^{118}, identified by anti-phosphotyrosine specific antibodies. Western blot analysis of Paxillin tyrosine phosphorylation reveals that HGF induces Paxillin phosphorylation of both Tyr^{31} and Tyr^{118} in control MDCK cells (Figure 4B). Surprisingly, basal phosphorylation of Paxillin in both residues is enhanced in cells expressing Dgkα-DN, and is not further affected by HGF stimulation (Figure 4B).

In summary, these data demonstrate that upon minutes of HGF stimulation, activation of Dgkα is required for the formation of membrane ruffles and for the succeeding remodelling of Paxillin- and Vinculin-containing focal complexes.

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(B) Dgkα localization in T-cells.

Project aim/objectives.

The aim of this project is to investigate the role of Dgkα in the XLP disease, characterized by a loss of function mutation of the gene SAP. In particular, I analyzed the localization of Dgkα in T cells upon stimulation of TCR.

Background.

The magnitude and specificity of cellular responses are dictated by a delicate balance between positive and negative signals that are generated after receptor stimulation. Although the mechanisms that promote the intracellular generation of signals have been extensively analyzed, those leading to negative regulation of signal generation remain largely undefined. Dgks generate PA through the phosphorylation of DAG. Since DAG is a well known lipid second messenger that is rapidly generated in response to receptor stimulation, Dgk activation may be related directly to the termination of DAG-derived signals.

Dgkα is the major isoform in T lymphocytes, and experiments using T cell lines have shown Dgkα translocation to the plasma membrane following TCR engagement (Sanjuan et al., 2001, Sanjuan et al., 2003). Dgkα activation is also required for IL-2-regulated cell cycle entry (Flores et al., 1996). Recent studies show that *in vivo* up-regulation of Dgkα results in T cell anergy (Olenchock et al., 2006), while its down-regulation results in a defect of T cell anergy (Zha et al., 2006).

The activation of immune cells is controlled through the balance of signals triggered by a wide range of cell surface receptors, including activating receptors (in particular the “immunoreceptors”), co-stimulatory receptors and inhibitory receptors. Cytokine receptors, receptors related to tumor necrosis factor (TNF) receptors, Toll-like receptors and SLAM-related receptors also influence immune cell activation (Veillette et al., 2006).

In recent years, there has been accumulating evidence implicating SLAM family receptors and a group of associated intracellular adaptors, the SLAM-associated protein (SAP)-related adaptors, in immune regulation (Veillette et al., 2006). The SAP family comprises three members: SAP,

Edwig's sarcoma-associated transcript-2 (EAT2) and, in rodents, EAT2-related transducer (ERT). All three members are composed of an SH2 domain and a short C-terminal tail (Veillette, 2006). SAP is expressed in T cells, NK cells, NKT cells, eosinophils, platelets and some B cells (Sayos et al., 1998; Sayos et al., 2000; Nichols et al., 2005; Munitz et al., 2005; Morra et al., 2005; Nanda et al., 2005). SAP is mutated in X-linked lymphoproliferative (XLP) disease, a human immunodeficiency disease characterized by a deregulated immune response to infection to Epstein-Barr virus (Sayos et al., 1998; Coffey et al., 1998; Latour et al., 2001). Typically, patients with XLP show an uncontrolled EBV-induced *infectious mononucleosis*, which leads to lymphoproliferation, organ failure and death (Nichols et al., 2005). In some cases, they develop hypogammaglobulinaemias, *common variable immunodeficiencies* (CVIDs) and lymphomas. The SAP mutations found in XLP are diverse, ranging from large deletions to point mutations in the gene. As a result of these alterations, the SAP polypeptide is absent, unstable or functionally inert (Veillette, 2006).

A close cooperation between our groups and Ornella Parolini's has provided preliminary results which strongly support the hypothesis that SAP in T cells may interact with the Dgkα protein. Studies *in vitro* report that T cell lines derived from XLP patients show defective proliferation, activation and cytokine production. It is tempting to speculate that the reduced T cell response observed in XLP patients could depend on failure to down-regulate Dgkα.

We have shown that, upon TCR and SLAM engagement, Dgkα activity is inhibited in a SAP-dependent manner, but the mechanism involved in this inhibition has not yet been understood. Here I analyzed the localization of Dgkα in resting T cells and co-stimulated either with TCR and CD28 or TCR and SLAM.

Experimental plan and methods.

Cell culture. Jurkat cells and PBL were cultured in high glucose RPMI GlutaMAX^{TM} medium (Gibco), supplemented with 10% fetal bovine serum (Gibco) and antibiotic-antimicotic solution (Sigma), in humidified atmosphere with 5% CO_2 at 37°C.

PBL isolation. Human lymphocytes were isolated from venous blood by Ficoll-Hystopaque separation method, a procedure for isolating human peripheral blood mononuclear cells (lymphocytes and monocytes) from a Buffy Coat.

Reagents. Anti-CD28 was from Ancell, anti-CD3 (OKT3) was a gift from Riccardo Mesturini, anti-SLAM from Tamas Schweighoffer, anti-Dgkα C20 from Santa Cruz, Alexa Fluor 546 Phalloidin from Molecular Probes. PP2, Src inhibitor, was purchased from Alexis, U-73122, PLC inhibitor and Wortmannin, PI 3-K inhibitor, from SIGMA, BAPTA-AM, an intracellular calcium chelator, from Invitrogen.

Expression vectors, transfections and infections with retroviral vectors. YFP-Dgkα-WT (wild type) was obtained by cloning Dgkα in pcDNA 6.2/N-YFP-DEST (Invitrogen) using Gateway kit (Invitrogen) according to manufacturer's instructions.

RNA interference. siRNAs against human SAP were chemically synthesized as double-strand RNA (Ambion). Sequences were as follows: S1 sense CGAGGAUUGGGAAAAAGUAtt antisense UACUUUUUCCCAAUCCUCGtt; S2 sense GGUUCUUGGAGUGCUGAGAtt antisense UCUCAGCACUCCAAGAACCtg. A GAPDH scramble siRNA (Ambion) was used as negative control.

Immunofluorescence. Cells were seeded on poly-L-Lysine (Sigma) coated glass coverslips in 24-well plates for 1 hour, then stimulated with 10 μg/ml of each stimulating antibody for 1 hour, in case with 10 μM PP2, 5 μM U73122, 100 nM Wortmannin and 10 μM BAPTA-AM. Cells were then processed as described before. Confocal images were acquired with the Leica confocal microscopy TSP2 and LCS Leica confocal software.

Results.

SAP is required for Dgkα membrane translocation upon TCR and SLAM co-stimulation.

T lymphocyte activation is triggered by stimulation of the antigen receptor in concert with co-stimulatory molecules through a complex signalling cascade, in which increases in membrane DAG and cytosolic calcium concentration are essential events (Kane et al., 2000).

It has been previously shown that, upon TCR and CD28 co-stimulation, obtained by treatment with stimulating antibodies, Dgkα translocates from cytosol to the plasma membrane (Sanjuan et al., 2003).

I initially observed the intracellular localization of Dgkα in resting Jurkat cells and in cells stimulated with TCR and CD28 or SLAM. Figure 5 shows that, upon 1 hour of stimulation, both treatments induce a complete translocation of the YFP-tagged protein to the plasma membrane (Figure 5B, panels c and d), indicating that also SLAM co-stimulation can regulate Dgkα localization in Jurkat cells. The stimulation of SLAM without TCR triggering has no effect on Dgkα localization.

SAP-related adaptors show a highly specific association with tyrosine-based motifs in the cytoplasmic domain of SLAM family receptors through their SH2 domains. The prototype of the SAP family, SAP, promotes tyrosine phosphorylation signals triggered by SLAM family receptors resulting from its ability to recruit FynT, a Src-related protein tyrosine kinase (Veillette 2006).

In order to assess the role of SAP in the membrane localization of Dgkα, I down-regulated the endogenous SAP by the expression of siRNAs against SAP. Two siRNAs were designed (S1, S2) and transiently co-transfected in Jurkat cells with GFP-SAP. The western blot shows that both siRNAs are effective in knocking down GFP-SAP protein, while negative control siRNA (scramble) does not affect GFP-SAP expression (Figure 5A). I then co-transfected YFP-Dgkα and scramble siRNA or S1 or S2. While Dgkα localizes to the plasma membrane upon TCR and CD28 co-stimulation in presence of both siRNAs (Figure 5B, panels c and d), the presence of S1 and S2 siRNAs completely impairs Dgkα translocation induced by TCR and SLAM co-stimulation (Figure 5C, panels c, d and data not

shown), indicating that SAP is required for the recruitment of Dgkα upon TCR and SLAM triggering.

These data suggest that distinct mechanisms regulate the localization of Dgkα upon different co-stimuli and that SAP is involved only in the signalling pathway downstream of SLAM receptor family.

Lymphoid cells express high level of endogenous Dgkα, so I observed the intracellular localization of the endogenous protein in peripheral blood lymphocytes (PBL). Similarly to what observed in Jurkat cells, Dgkα translocates to the plasma membrane upon stimulation of TCR plus CD28 and TCR plus SLAM (Figure 6A). I analyzed the images acquired and I scored the cells for presence of Dgkα in the plasma membrane. As one can see in the bar graph in figure 6B, the percentage of cells featuring Dgkα in the membrane raises from less than 30% in control cells to about 70% in PBL stimulated either with TCR and CD28 or TCR and SLAM, indicating that the YFP fusion protein has the same behaviour as the endogenous one.

Src-family kinases and intracellular calcium increase are required for Dgkα translocation induced by TCR and SLAM co-stimulation.

Sanjuan et al. (2001) demonstrated that, upon stimulation of the ectopic expressed carbachol receptor, Dgkα rapidly translocated to the plasma membrane. They also showed that carbachol-induced translocation of GFP-Dgkα was prevented by pre-treatment of transfected cells with the PLC inhibitor U-73122 or with the calcium chelator BAPTA, indicating that calcium signals are necessary for Dgkα recruitment to the plasma membrane. Moreover, enzyme translocation in response to carbachol was blocked by pre-treatment of the cells with the tyrosine kinase inhibitor herbimycin A, which interferes with early T cell signalling events by inhibiting Lck and ZAP-70 function.

I then investigated if calcium generation and activation of tyrosine kinases are also involved in Dgkα recruitment induced by more physiological stimuli, such as TCR and CD28 and TCR and SLAM co-stimulations.

As described before, Dgkα translocates to the plasma membrane in stimulated cells (Figure 7, panel c and k). In agreement with Sanjuan's results, U-73122 and BAPTA abolished Dgkα recruitment induced by both co-stimulations, while PP2 was able to block only TCR + SLAM induced Dgkα recruitment and had no effect on TCR and CD28 co-simulation, indicating that probably the co-stimulatory receptors elicit their function through different signalling cascades and SLAM is more dependent on Src family kinases than CD28 (Figure 7). Acquired images were observed and cells were scored for presence of Dgkα on plasma membrane. The bar graph in figure 8A shows that the percentage of cells featuring plasma membrane localized Dgkα raises from about 40% in control cells to more than 80% in stimulated cells, while in presence U-73122 and BAPTA it is reduces to almost the control value. The same results were obtained observing endogenous Dgkα localization in PBL upon the same treatments (data not shown and Figure 8B). Finally, phosphatidylinositol 3-kinase (PI 3-

K) is not involved in Dgkα translocation, since its inhibitor Wortmannin does not impair Dgkα membrane localization upon both stimulations (Figure 8B).

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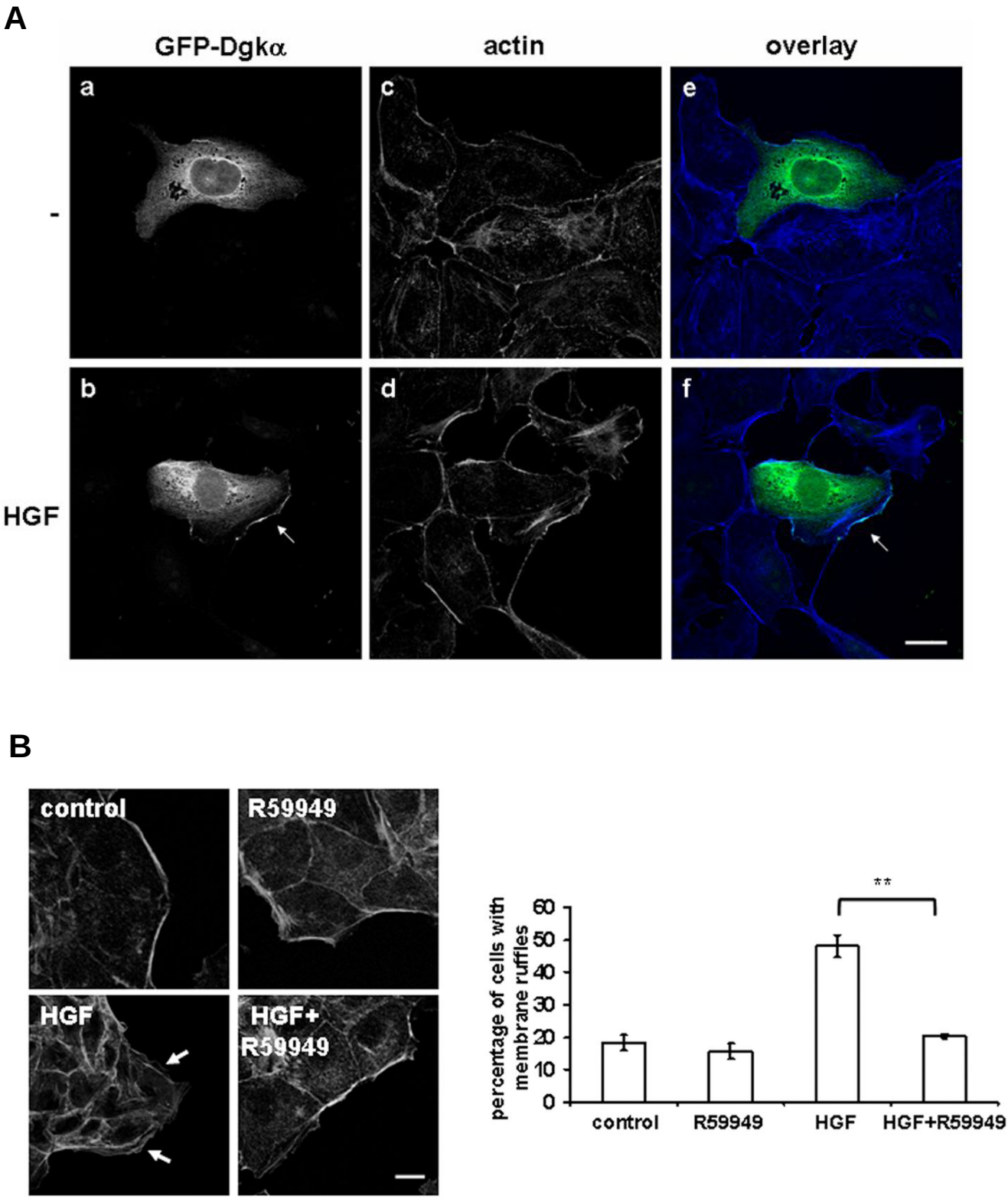

Figure 1. (A) MDCK cells were transiently transfected with GFP-Dgkα, starved overnight in 0% FBS medium, stimulated with HGF 10 ng/ml for 15 minutes, fixed and stained for actin. Scale bar = 8 μm (B) MDCK cell colonies were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for actin. Scale bar = 16 μm. Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles (arrows). The percentage of cells with membrane ruffles was calculated. Means of three experiments with standard errors are shown. ** = $p<0.005$.

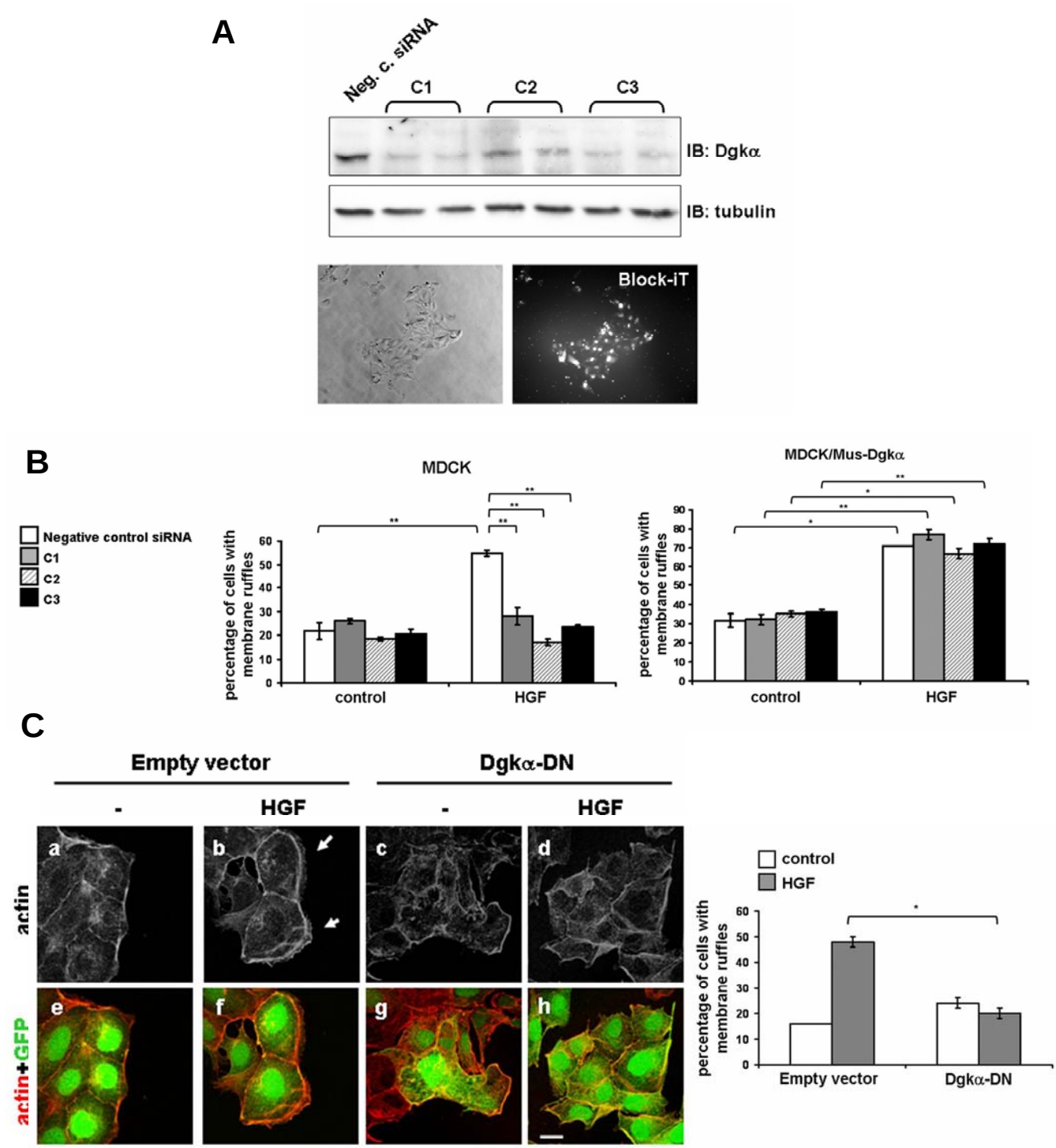

Figure 2. (A) Lysates of MDCK cells transiently transfected with negative control siRNA or canine Dgkα siRNAs C1, C2 and C3 were separated by SDS-PAGE and after blotting were probed for Dgkα and tubulin. MDCK cell colonies were transfected with Block-IT fluorescent siRNA to evaluate the efficiency of transfection. (B) MDCK and MDCK/Mus-Dgkα were transiently transfected with negative control siRNA or canine Dgkα siRNAs C1, C2 or C3, starved overnight in 0% FBS medium and treated with HGF 10 ng/ml for 15 minutes. Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles. The percentage of cells with membrane ruffles was calculated. Means of three experiments with standard errors are shown. * = $p<0.05$, ** = $p<0.005$. (C) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for actin (red, panels a,b,c,d). The arrows indicate membrane ruffles in empty vector-infected cells. Scale bar = 16 μm. Representative pictures are shown. 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 errors are presented. * = $p<0.05$.

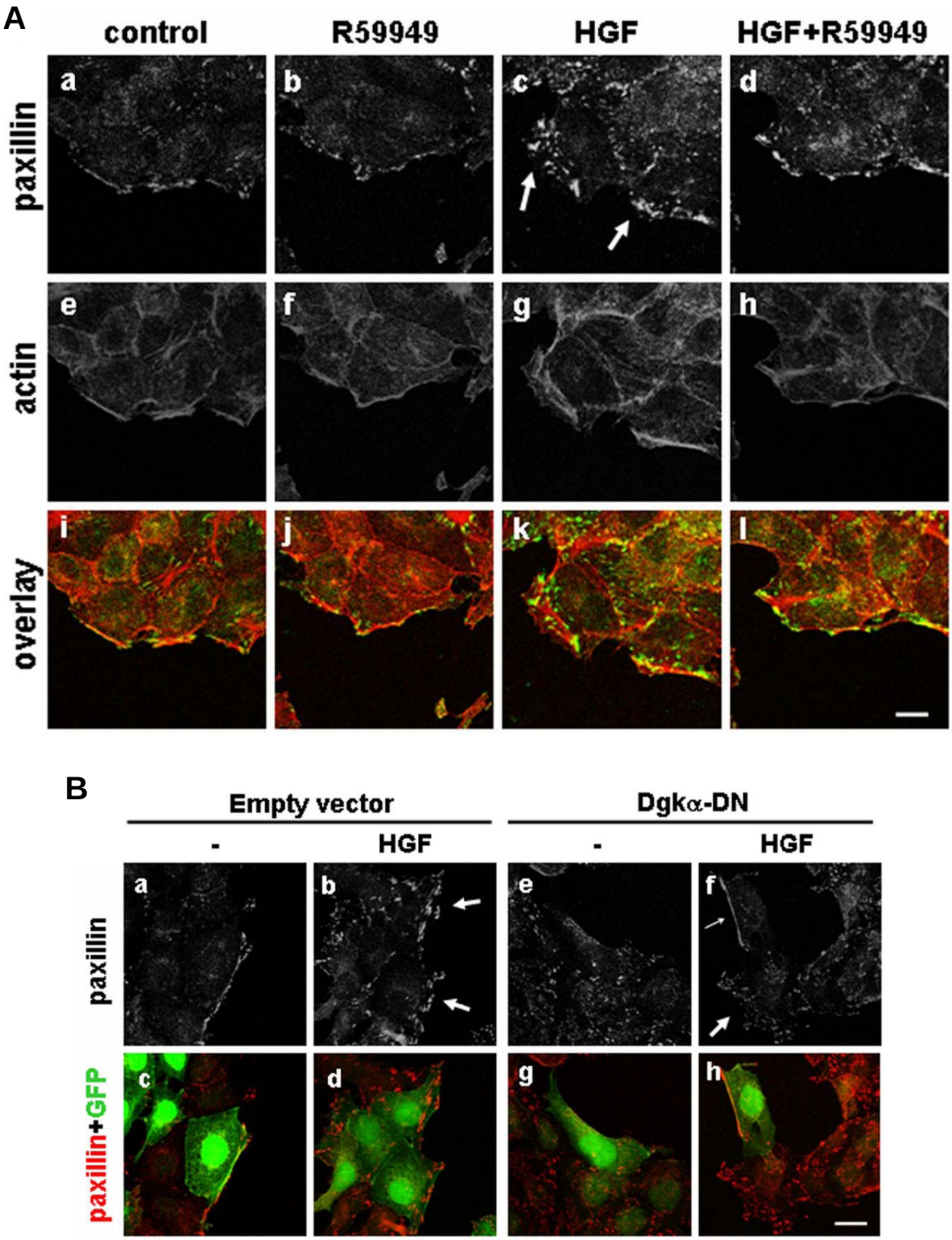

Figure 3. (A) MDCK cell colonies were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for Paxillin (green, panels a,b,c,d) and actin (red, panels e,f,g,h). Representative pictures are shown. Scale bar = 16 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for Paxillin (red, panels a,b,e,f). Thick arrows indicate Paxillin localization at focal adhesions in the areas 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.

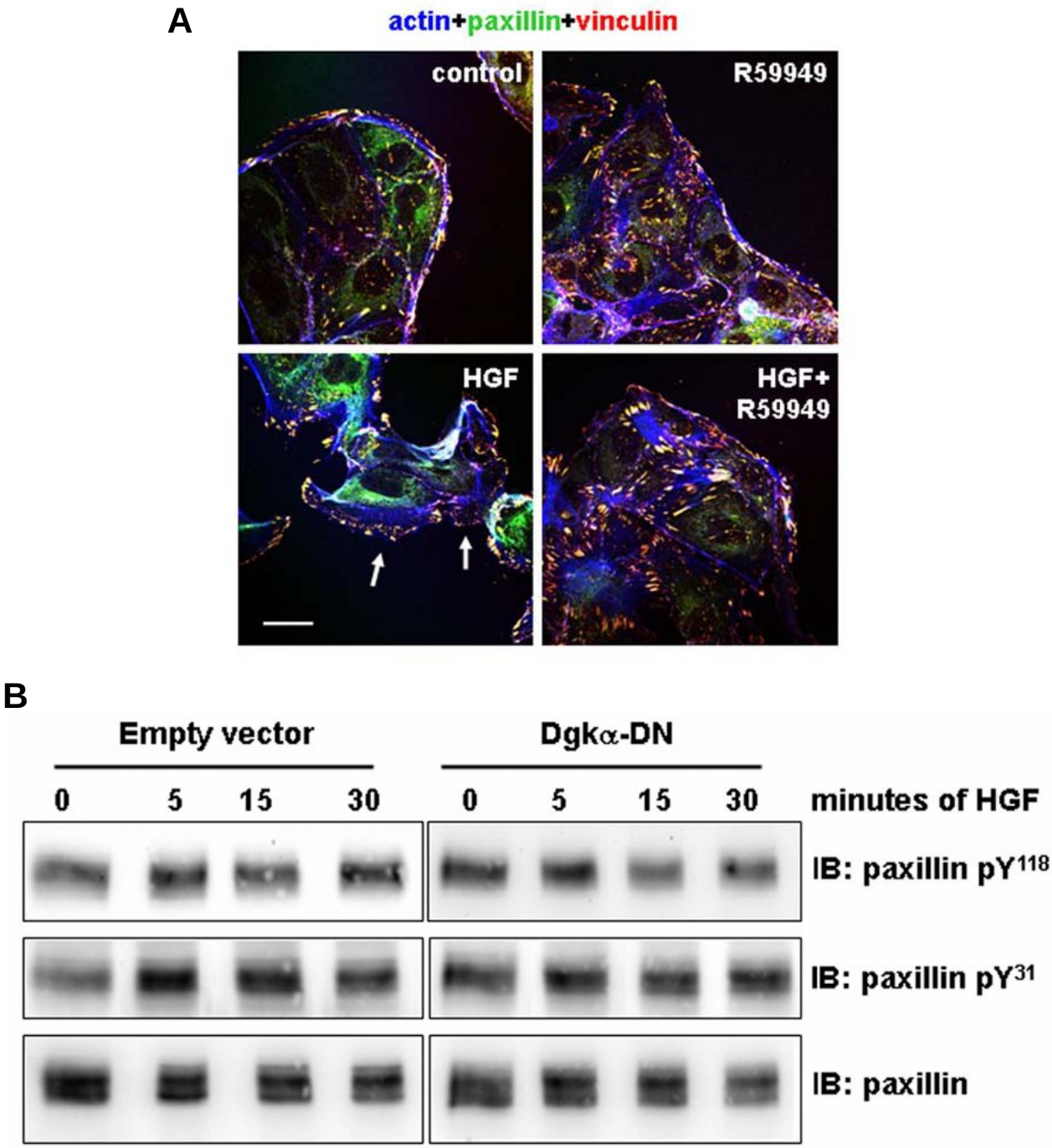

Figure 4. (A) MDCK cell colonies were treated as described in figure 3A, fixed and stained for Paxillin (green), Vinculin (red) and actin (blue). Representative pictures are shown. Scale bar = 16 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium and treated with HGF 50 ng/ml for 15 minutes. Whole cell lysates were separated by SDS-PAGE and probed with anti-Paxillin pTyr31 and pTyr118 and anti-Paxillin.

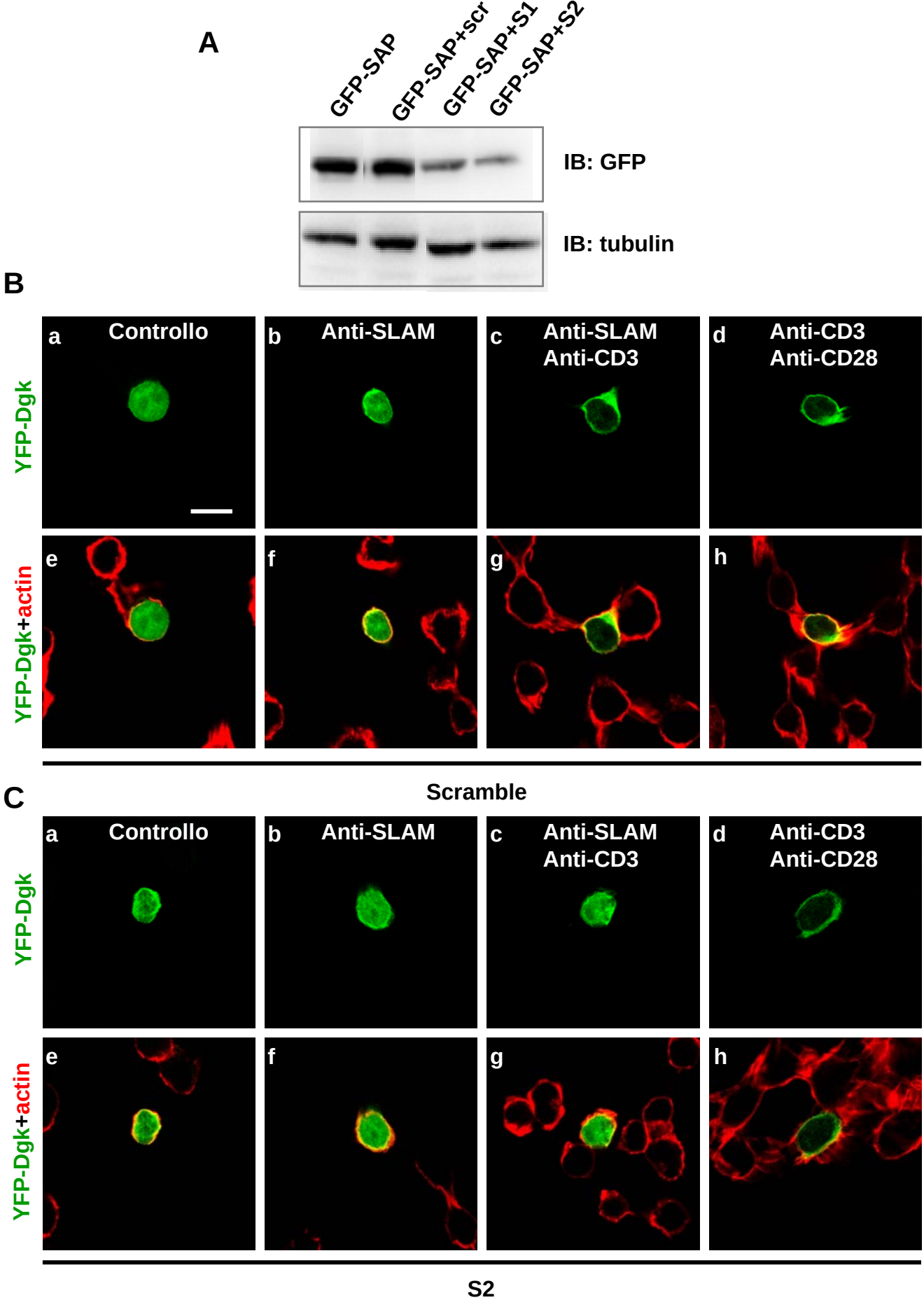

Figure 5. (A) Lysates of Jurkat cells transiently transfected with GFP-SAP and scramble siRNA or SAP siRNAs S1and S2 were separated by SDS-PAGE and after blotting were probed for SAP and tubulin. Jurkat cells tranfected with scramble (C) or SAP siRNA S2 (D) and YFP-Dgkα were treated with 10 μg/ml of each indicated antibody for 1 hour, then fixed and stained for actin (red). Scale bar = 7 μm

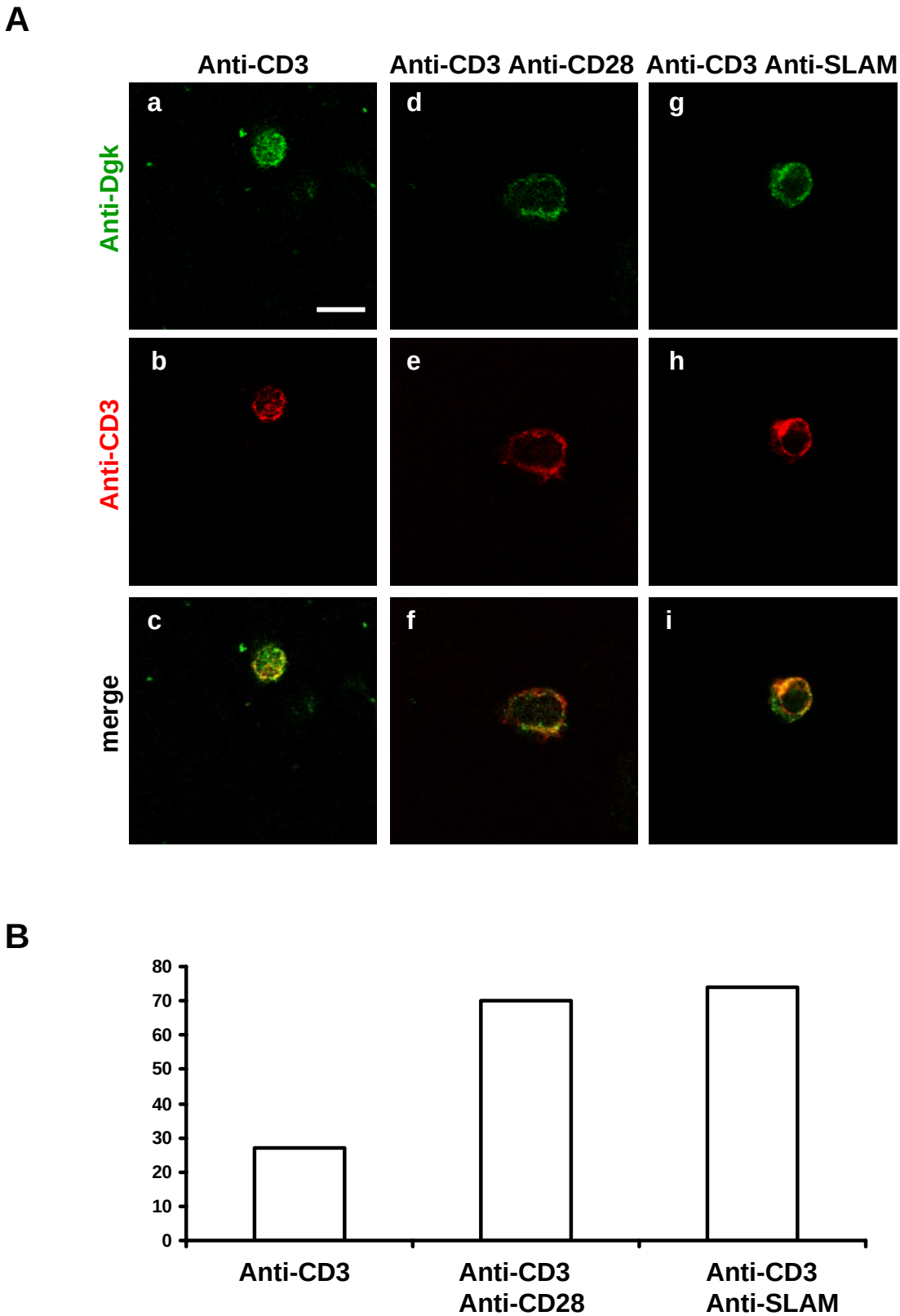

Figure 6. (A) PBL were treated as described in figure 5, fixed and stained for Dgkα (green) and CD3 (red). Scale bar = 7μm. (B) Confocal acquired images were observed and cells were scored for presence of Dgk α on the plasma membrane.

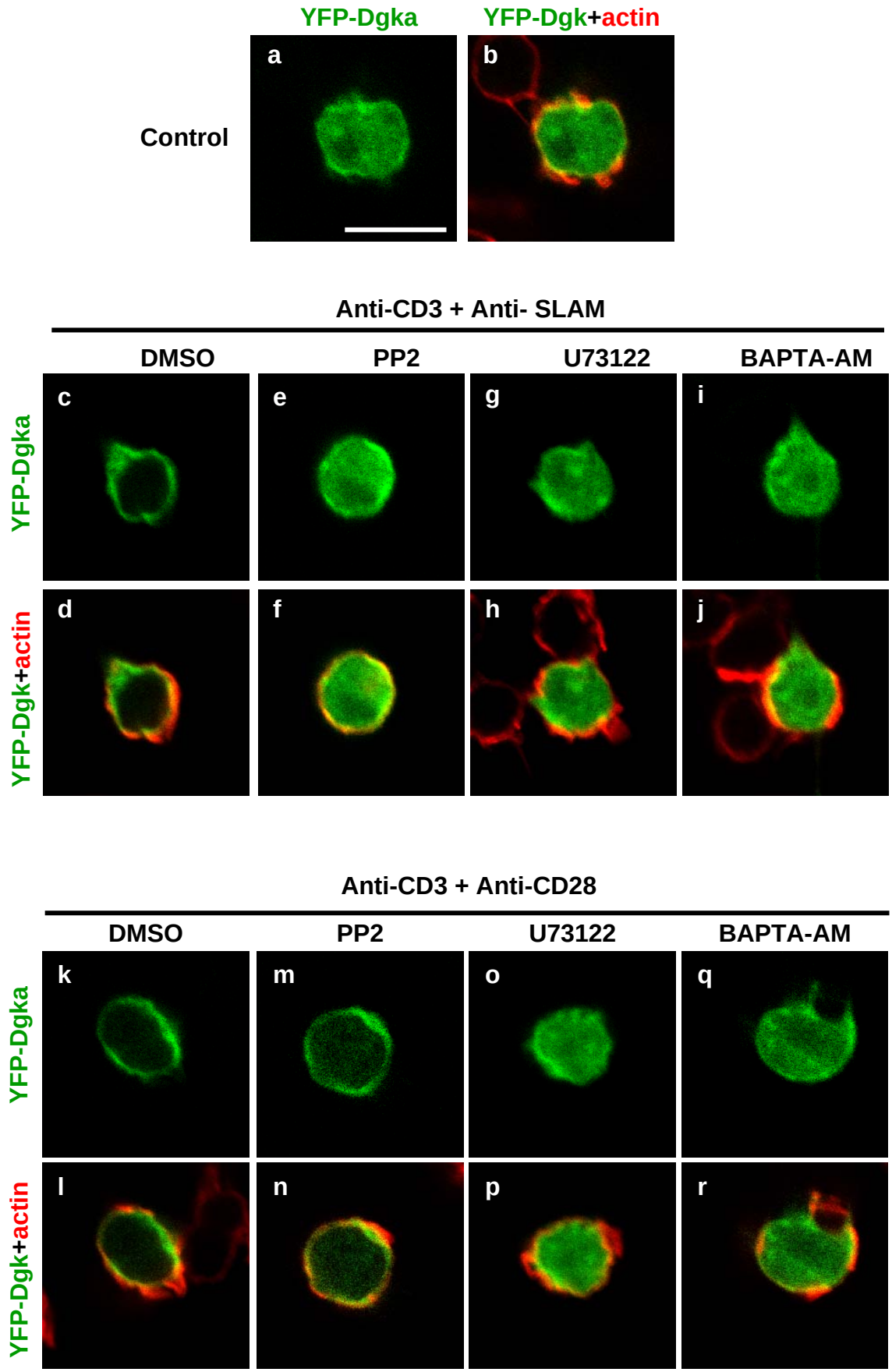

Figure 7. Jurkat cells tranfected with YFP-Dgkα were treated with 10 μg/ml of each indicated antibody in presence or in absence of 10 μM PP2, 5μM U73122 and 10μM BAPTA-AM for 1 hour, then fixed and stained for actin (red). Scale bar = 7 μm

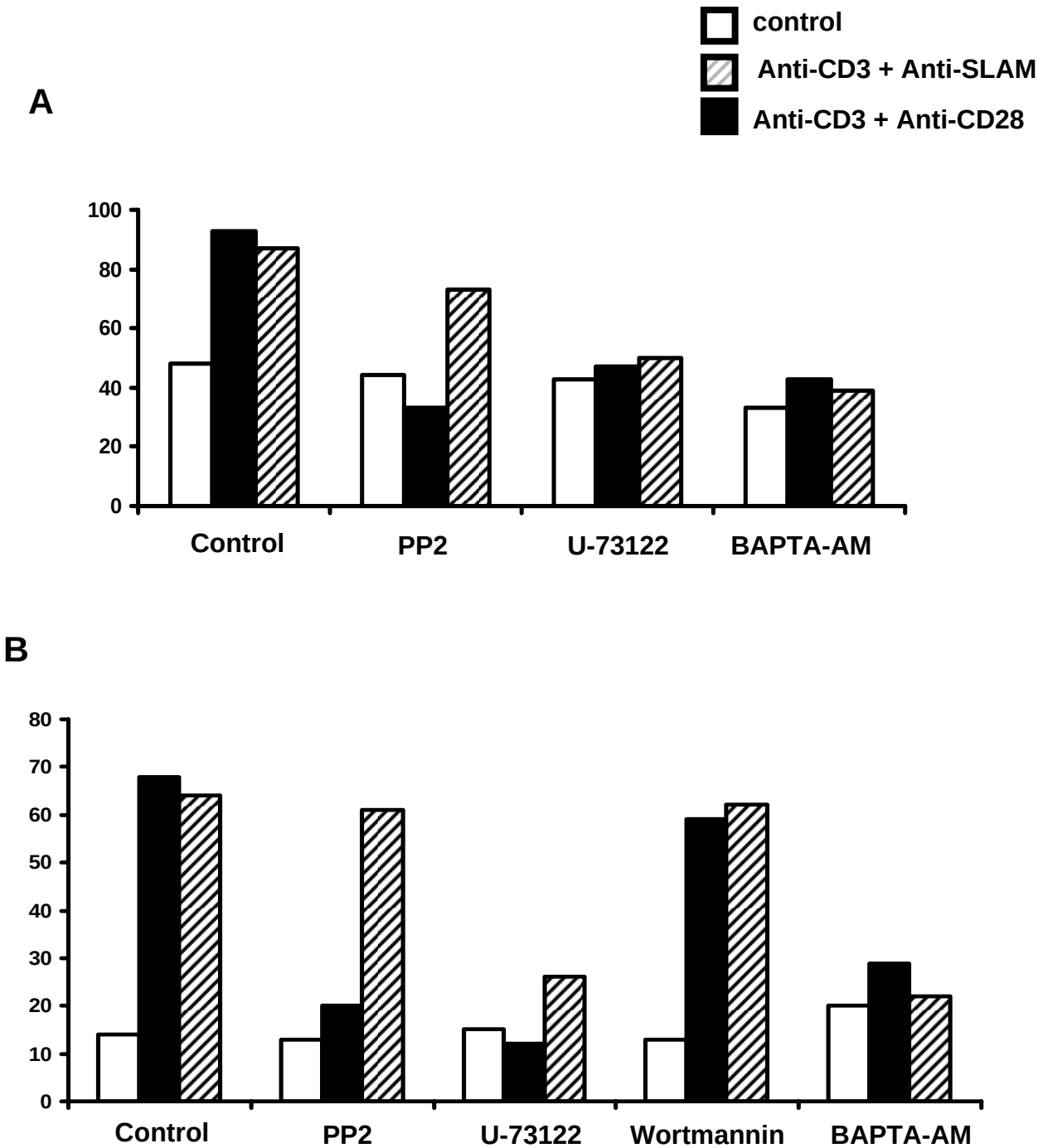

Figure 8. (A) Jurkat cells tranfected with YFP-Dgkα were treated as indicated in figure 7. (B) PBL were treated as (A) and with 100nM Wortmannin. Confocal acquired images were observed and cells were scored for presence of Dgkα on the plasma membrane.

Tabella B.4a - Stage presso enti esterni

Gli "stage" si intendono come periodi di apprendistato lavorativo e/o di ricerca presso enti/aziende italiane o straniere. Ogni studente deve compilare le schede rispettando il formato (Times New Roman 12) e l'impaginazione in quanto i dati saranno inclusi come tali nelle schede da inviare al nucleo di valutazione dell'Ateneo.

Studente	Ciclo	Ente presso cui si è svolto lo stage	Periodo [in mesi]	Argomento

{Aggiungere le righe necessarie}

B.5 Percorso formativo

Tabella B.5b - Corsi tenuti (in Italia o all'estero) per il Dottorato

Lo studente deve elencare tutti i corsi didattici (per es. quello di Inglese e/o di Statistica), monografici (per es. quello sulla RNAi), e seminari ai quali ha partecipato. Rispettare il formato e l'impaginazione come sopra. Per i seminari inserire la data nella colonna "Numero di ore", e la dicitura "Firme di frequenza" in quella di Modalità di accertamento.

Corso di insegnamento o seminario (+ località)	Docente (+ Affiliazione)	Numero di ore	Modalità di accertamento {Esame, Progetto, Seminario, Relazione, Altro (specificare)}
Inglese	C. Irving	30	Firme di frequenza
Statistica	C. Magnani, Università Piemonte Orinetale	8	
VIII Seminario Leica: microscopia confocale e sue applicazioni (Castel Gandolfo, Roma)		20-21-22 giugno 2007	Attestato di frequenza
RNAi	Defilippi e Caselle, Università di Torino;	10 e 17 gennaio 07	Firme di frequenza
Nuove frontiere dei biomateriali: applicazioni cliniche	R. Barbucci, Università di Siena	29-11-06	Firme di frequenza
Autoantibodies in systemic sclerosis: from clinical subsets to pathogenitic functions	C. Chizzolini, Geneva University Hospital	12-01-07	Firme di frequenza
Nuovi ruoli per le proteine adattatrici p130Cas e p130Cap nella trasformazione tumorale e nel carcinoma della mammella	P. Defilippi, Università di Torino	25-01-07	Firme di frequenza
Genes therapy strategies for phenylketonuria	T. Beat, University of Zurich	01-02-07	Firme di frequenza
Fragile X syndrome from RNA metabolism impairment to spine dysmorphogenesis	C. Bagni, University of Rome, Tor Vergata	22-02-07	Firme di frequenza
Protein mycroarrays development of new supports for improved sensitivity	M. Cretich, ICRM-CNR Milano	14-03-07	Firme di frequenza
Drosophila as a model for aging and cancer	D. Bohmann, University of Rochester	16-03-07	Firme di frequenza

Marcatori farmacogenomici nel carcinoma colorettale: quail prospettive per una terapia personalizzata?	E. Mini	29-03-07	Firme di frequenza
BRET and FRET: new technologies for mapping receptor/G protein interaction	Dipartimento di farmacologia, università di Milano	30-03-07	Attestato di partecipazione
Pathogenic viruses: smart manipulators of the interferon system	O. Haller, Istituto di Virologia, Università di Friburgo	19-04-07	Firme di frequenza
The regulation of hematopoietic stem cells by smad signalling	S. Karlsson, Lund University, Sweden	25-05-07	Firme di frequenza
Translating basic science into therapeutic strategies for Shwachman Diamond Syndrome	S.R. Ellis, University of Louisville (USA)	28-05-07	Firme di frequenza
Relazione tra funzione e struttura delle macromolecole biologiche	M. Milanesio	12-04-07	Firme di frequenza
Corso di radioprotezione	M. Brambilla	12-06-07	Firme di frequenza
Sindromi autoinfiammatorie	A. Martini, Università di Genova	4-06-07	Firme di frequenza
Bioinformatic tools for the analysis of UTRs and for the prediction of alternative spliced transcripts	F. Mignone, Università di Milano	21-06-07	Firme di frequenza

{Aggiungere le righe necessarie}

Sezione C: Risultati conseguiti nei cicli attivi dello stesso dottorato

Nelle tabelle, i valori richiesti si riferiscono alla carriera totale del Dottorando. Ogni studente deve inserire i dati richiesti relativi al ciclo di apparteneza. Rispettate formato ed impaginazione

Tabella C.1 - Studenti iscritti

Ciclo XXII

Nome e Cognome	Anno nascita	Laureato/a presso Università di …	Borsa (SI/NO)	Se senza borsa, forma di sostegno economico	Mesi totali trascorsi all'estero	Numero pubblicazioni	Numero di partecipazioni a conferenze
Elena Rainero	**1982**	**Piemonte Orientale**	**sì**		**0**	**2**	**4**

{Aggiungere le righe necessarie}

(1) Allegare l'elenco dei **soggiorni**

Studente	Ente	Luogo	Anno	Docente/ricercatore di riferimento nell'ente ospitante (*)

(*)Se applicabile.

(2) Allegare l'elenco delle **pubblicazioni (**di cui almeno un dottorando è co-autore)

G. Baldanzi, S. Cutrupi, F. Chianale, V. Gnocchi, **E. Rainero**, P.Portporato, N. Filigheddu, W.J. van Blitterswijk, O. Parolini, F.Bussolino, F. Sinigaglia, A. Graziani (2007). Diacylglycerol kinase alpha phosphorylation by Src on Y335 is required for activation, membrane recruitment and HGF induced cell motility. Oncogene, in press

F. Chianale, S. Cutrupi, **E. Rainero**, G. Baldanzi, P.E. Porporato, S. Traini, N. Filigheddu, V. Gnocchi, M.M. Santoro, O. Parolini, W.J. van Blitterswijk, F. Sinigaglia, A. Graziani (2007) Diacylglycerol kinase alpha mediates HGF-induced epithelial cell scatter by regulating Rac activation and membrane ruffling. Mol. Biol. Cell in press.

(3) Allegare l'elenco delle **conferenze/workshop (**a cui almeno un dottorando ha partecipato)

Studente	Conferenza/Workshop	Luogo	Anno	Studente relatore {SI, NO}
Elena Rainero	Segnali di calcio in Piemonte	Novara	2007	no
Elena Rainero	Phosphoinositides on the slope	Fara San Martino	2007	no
Elena Rainero	Workshop on cell migrationn	Milano	2007	no
Elena Rainero	VIIII seminario Leica: microscopia confocale e sue applicazioni	Castel Gandolfo	2007	no

Ciclo XXI

Nome e Cognome	Anno nascita	Laureato/a presso Università di …	Borsa (SI/NO)	Se senza borsa, forma di sostegno economico	Mesi totali trascorsi all'estero	Numero pubblicazioni	Numero di partecipazioni a conferenze

{Aggiungere le righe necessarie}

(1) Allegare l'elenco dei **soggiorni**

Studente	Ente	Luogo	Periodo temporale	Docente/ricercatore di riferimento nell'ente ospitante (*)

(*)Se applicabile.

(2) Allegare l'elenco delle **pubblicazioni (**di cui almeno un dottorando è co-autore)

(3) Allegare l'elenco delle **conferenze/workshop (**a cui almeno un dottorando ha partecipato)

Studente	**Conferenza/Workshop**	**Luogo**	**Anno**	**Studente relatore** {SI, NO}