Keratin 8 phosphorylation regulates keratin reorganization and migration of epithelial tumor cells

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Keywords: Intermediate filaments, mitogen activated protein kinases, sphingosylphosphorylcholine, gastric cancer cells, pancreatic cancer cells
Summary

Cell migration and invasion are largely dependent on the complex organization of the various cytoskeletal components. Whereas the role of actin filaments and microtubules in cell motility is well established, the role of intermediate filaments (IF) in this process is incompletely understood.

Organization and structure of the keratin cytoskeleton consisting of heteropolymers of at least one type 1 and one type 2 IF, are in part regulated via posttranslational modifications. In particular, phosphorylation events influence the properties of the keratin network. Sphingosylphosphorylcholine (SPC) is a bioactive lipid with the exceptional ability to change the organization of the keratin cytoskeleton, leading to reorganization of keratin filaments, increased elasticity, and subsequently increased migration of epithelial tumor cells.

Here we investigate the signaling pathways that mediate SPC-induced keratin reorganization and the role of keratin phosphorylation in this process. We establish that the MEK-ERK signaling cascade regulates both SPC-induced keratin phosphorylation and reorganization in human pancreatic and gastric cancer cells and identify Ser^{431} in K8 as the critical residue whose phosphorylation is required and sufficient to induce keratin reorganization and consequently enhanced migration of human epithelial tumor cells.
Introduction

Cell migration and invasion are markedly dependent on the complex organization of the cytoskeleton (Ballestrem et al., 2000). The cytoskeleton of epithelial cells is a network of three major classes of filamentous biopolymers: microfilaments, microtubules, and intermediate filaments. Intermediate filaments are composed of a large family of cell-specific proteins that organize to form 10 nm filaments sharing sequence homology and structural features. Among the cytoplasmic intermediate filament proteins, keratins are expressed preferentially in epithelial cells (Coulombe and Omary, 2002; Fuchs and Weber, 1994) and constitute nearly 5% of the total protein in these cells (Omary et al., 1998). Keratin filaments are obligate heteropolymers of at least one type I (relatively acidic keratins K9-K28, K31-K40) and one type II keratin (relatively basic keratins K1-K8, K71-K86 (Schweizer et al., 2006)). These filaments are usually organized into bundles, the so called tonofibrils, which form cage-like structures around the nucleus and extend from the perinuclear region to the cell periphery (Hatzfeld and Franke, 1985).

K8 and K18 are the major components of intermediate filaments of simple epithelia as found in intestine, liver and exocrine pancreas (Coulombe and Omary, 2002; Fuchs and Weber, 1994). The expression pattern of these proteins is generally persistent in carcinomas arising from tissues that normally express K8 and K18 (Oshima et al., 1996). Keratins play a crucial role in maintaining the structural integrity and the mechanical properties of cells and thereby protect cells from a variety of environmental insults (Yamada et al., 2002). Furthermore, they are major determinants for the mechanical features of the cytoplasm and the nucleus (Fuchs and Cleveland, 1998; Maniotis et al., 1997).

The structure and function of keratins are very likely regulated through posttranslational modifications, particularly phosphorylation on serine residues within the so-called ‘head’ (N-terminal) and/or ‘tail’ (C-terminal) non-α-helical end domains (Fuchs and Cleveland, 1998; Omary et al., 2006). Ser52 is the major phosphorylation site of human K18 in vivo. This site has been implicated in increased keratin solubility and altered polymerization (Ku and Omary, 1994; Liao et al., 1995b), keratin reorganization (Ku et al., 1999), apoptosis (Caulin et al., 1997) and cellular stress (Omary et al., 1998). Increased phosphorylation of K18 has also been implicated in the reorganization of keratin filaments in hepatocytes treated with protein phosphatase inhibitors (Toivola et al., 1998).
Ser\(^{431}\) is a major in vivo phosphorylation site in human K8. Ser\(^{431}\) is phosphorylated by MAP kinases in response to activation of the EGFR (Omary et al., 1998). Phosphorylation at this site has also been described during hyperosmotic stress, whereas hypo-osmotic stress leads to dephosphorylation at K8-S\(^{431}\) (Tao et al., 2006), and also occurs in human and mouse livers upon injury resulting in Mallory-Denk body formation (Stumptner et al., 2000) or during mouse liver and gallbladder injury induced by a high-fat diet (Tao et al., 2003).

Sphingosylphosphorylcholine (SPC) is a naturally occurring bioactive lipid that acts as an intracellular and extracellular signaling molecule in numerous biological processes including proliferation (Seufferlein and Rozengurt, 1995), cell migration (Boguslawski et al., 2000), wound healing (Wakita et al., 1998), and differentiation (Kleger et al., 2007). Similar to other bioactive lipids such as lysophosphatidic acid (LPA) or sphingosine-1-phosphate, many of its actions are mediated by the activation of a subfamily of low and high affinity G protein coupled receptors (An et al., 1995; Meyer zu Heringdorf et al., 2002).

Previously, we have shown that SPC is one of the few naturally occurring compounds known so far that can induce a perinuclear reorganization of the keratin cytoskeleton in human pancreatic cancer cells. This reorganization is accompanied by keratin phosphorylation, including phosphorylation at K18-S\(^{52}\) and K8-S\(^{431}\), and an increase in cellular elasticity and enhanced migration of cancer cells through size limited pores (Beil et al., 2003). However, the precise downstream signaling mechanisms by which SPC induces keratin reorganization and the role of keratin phosphorylation in this process are as yet unknown.

Here we show that the MEK-ERK signaling cascade regulates both SPC-induced K8 phosphorylation at Ser\(^{431}\) and keratin reorganization in human pancreatic and gastric cancer cells. We identify Ser\(^{431}\) in K8 as the critical residue whose phosphorylation is required and sufficient to induce keratin reorganization and consequently enhanced migration of human epithelial tumor cells.
Results

Role of the ERK cascade for SPC-induced keratin reorganization

Previously we have demonstrated that SPC reorganizes the keratin cytoskeleton in Panc-1 and AGS human cancer cells from a branched phenotype into a perinuclear, ring-like formation and increases migration of epithelial tumor cells through size limited pores (Beil et al., 2003). These cell lines express K8 and K18 as their major keratins, as shown using a pan anti-keratin antibody and individual K8 and K18 antibodies (Fig. S1A). This effect of SPC is most likely mediated by a G protein coupled receptor. SPC interacts with S1P receptors 1-5, GPR4, and OGR1 with different affinities (Meyer zu Heringdorf et al., 2002). Both, pancreatic and gastric cancer cell lines express S1P1-5, GPR4, and OGR1 as determined by RT-PCR (Fig. S1B).

Activation of the ERK signaling cascade has been implicated in cell migration (Bove et al., 2008; Huang et al., 2004; Rajalingam et al., 2005). We have previously shown that SPC potently induces activation of ERKs in fibroblasts (Seufferlein and Rozengurt, 1994). SPC was also able to stimulate ERK activation in human pancreatic and gastric cancer cells reaching a maximum after 15 and 30 minutes of incubation in Panc-1 and AGS cells, respectively. ERK activation in Panc-1 and AGS cells in response to SPC was prevented by incubation of cells with the selective MEK inhibitors PD98059 or U0126 (Fig. 1). Interestingly, SPC failed to activate other signaling pathways in pancreatic cancer cells including the PI3-K/AKT, the PKC-PKD, and the PKM2 signaling pathways (Fig. S2).

ERKs also regulate keratin phosphorylation (Huang et al., 2004; Ku and Omary, 1997; Omary et al., 2006). Therefore, we determined whether ERK-mediated phosphorylation of keratins could play a role in SPC-induced keratin reorganization and migration. SPC induced perinuclear reorganization of both, endogenous keratin and overexpressed K8/K18 (Fig. 2A). Keratin perinuclear reorganization was confirmed by quantifying the fluorescence intensity distribution on the Y axis (Fig. 2B, 6E). SPC-induced keratin reorganization was completely prevented upon incubation of Panc-1 or AGS cells with the MEK inhibitors PD90859 or U0126, respectively (Fig. 3). Thus, activation of the MEK/ERK cascade appears to be critical for perinuclear keratin reorganization in response to SPC.
ERKs mediate SPC-induced phosphorylation of K8-S^{431}

Next we wanted to identify the site(s) in keratins that become phosphorylated in response to SPC. SPC stimulated phosphorylation of K8 at Ser^{431} in both epithelial cancer cell lines, a site that is also phosphorylated by active ERK (Ku et al., 2005; Omary et al., 1998). K8-S^{431} phosphorylation in response to SPC was prevented when cells were incubated with either MEK inhibitor (Fig. 4A). To determine the effect of ERK-induced K8 phosphorylation on the organization of the keratin cytoskeleton we performed immunocytochemistry using a phosphospecific pK8-S^{431} antibody. Using this antibody we detected intense pK8-S^{431} immunoreactivity exclusively upon incubation of cells with SPC, but not in unstimulated, control cells. pK8-S^{431} immunoreactivity was predominantly detectable in reorganized, perinuclear keratin filaments indicating that K8 phosphorylation strictly correlates with keratin reorganization (Fig. 4C). In the presence of U0126 or PD98059, the SPC-stimulated increase in Ser^{431} immunoreactivity was virtually abolished (Fig. 4D). Similarly, the SPC-stimulated increase in K8-S^{431} immunoreactivity was completely prevented when p42/p44 were depleted by specific siRNAs (Fig. 4E). Thus, SPC-induced K8-S^{431} phosphorylation requires ERK activity.

SPC induces keratin phosphorylation also at other sites. Incubation of cells with SPC increased the phosphorylation of K18-S^{52} in both pancreatic and gastric cancer cells. However, K18-S^{52} phosphorylation in response to SPC was not prevented by inhibition of the MEK-ERK signaling cascade with PD90859 or U0126, respectively (Fig. 4B). Collectively, these findings indicate that: 1) SPC stimulates ERK activity, 2) SPC-induced keratin reorganization requires ERK activity, 3) SPC-induced phosphorylation of K8-S^{431} is also dependent on ERK activity and 4) K8-S^{431} phosphorylation and keratin reorganization by SPC go hand in hand. These data suggest a relationship between K8-S^{431} phosphorylation and keratin reorganization in epithelial tumor cells.

Role of phosphorylation at K8-S^{431} and K18-S^{52} in SPC-induced keratin reorganization in human cancer cells

To examine whether keratin phosphorylation at K8-S^{431} or K18-S^{52} was required and/or sufficient for SPC-induced keratin reorganization in Panc-1 and AGS cells, we generated eCFP-tagged mutants of K8 and eYFP-tagged mutants of K18 that mimic phosphorylation at K8-S^{431} and K18-S^{52}, respectively (S→E), or exhibit a non phosphorylatable site (S→A). It has been shown previously that keratin phosphorylation affects its solu-
bility. Indeed, there was more K8-S\(^{431}\)E detectable in the cytosolic/soluble fraction compared to K8-S\(^{431}\)A and K8-WT (Fig. S2A).

Upon incubation of cells with SPC, endogenous as well as exogenously expressed K8 and K18 exhibited the typical pattern of “reorganized” keratin with a predominant perinuclear keratin organization in Panc-1 and AGS cells that was prevented in the presence of U0126 (Figs. 5A, S3A). When cells were transfected with an eCFP-K8-S\(^{431}\)A/K18-S\(^{52}\)A double mutant there was no detectable keratin reorganization in response to SPC (Figs. 5B, S3B). In marked contrast, transfection of cells with the K8-S\(^{431}\)E/K18-S\(^{52}\)E mutants resulted in a strictly perinuclear redistribution of these keratin mutants that was not further increased in presence of SPC. Incubation of K8-S\(^{431}\)E/K18-S\(^{52}\)E transfected cells with U0126 did not prevent the perinuclear organization of the transfected keratins (Figs. 5C, S3C). Thus, phosphorylation of K8 and/or K18 is sufficient to trigger keratin reorganization.

To determine whether and which of these phosphorylations were required and/or even sufficient to trigger perinuclear keratin reorganization we expressed K8 and K18 expression plasmids that contained only one keratin modification either in keratin 8 or keratin 18. Organization of K8-wt/K18-S\(^{52}\)E transfected Panc-1 or AGS cells was comparable to that of wild type keratins. Perinuclear organization of K8-wt/K18-S\(^{52}\)E was only detectable in the presence, but not in the absence of SPC (Figs. 5D, S3D). This SPC-induced perinuclear reorganization of K8-wt/K18-S\(^{52}\)E could be prevented by treatment of cells with U0126. Organization of the K8-wt/K18-S\(^{52}\)A mutant was comparable to that of wild type K8/K18 in the presence or absence of SPC (Figs. 3, 5E, S3E). Thus, K18-S\(^{52}\) phosphorylation was neither sufficient nor required for SPC-induced perinuclear keratin reorganization in epithelial cancer cells.

Transfection of Panc-1 or AGS cells with K8-S\(^{431}\)E/K18-wt resulted in marked perinuclear reorganization of keratin already in the absence of SPC. Incubation of cells with SPC did not further enhance perinuclear organization of K8-S\(^{431}\)E/K18-wt. Furthermore, incubation of cells with U0126 did not prevent the perinuclear organization of K8-S\(^{431}\)E/K18-wt (Figs. 6A, S4A). In turn, K8-S\(^{431}\)A/K18-wt did not exhibit perinuclear keratin organization either in the absence or in the presence of SPC. The selective MEK inhibitor U0126 had no effect on the subcellular organization of K8-S\(^{431}\)A/K18-wt (Figs. 6B, S4B).
Furthermore, there was no difference in the perinuclear organization of K8-S\textsuperscript{431}E/K18-wt, K8-S\textsuperscript{431}E/K18-S\textsuperscript{52}E or K8-S\textsuperscript{431}E/K18-S\textsuperscript{52}A indicating that phosphorylation of K18 at Ser\textsuperscript{52} is indeed not required for perinuclear keratin organization in human epithelial tumor cells (Figs 6C, S4C). Interestingly, transfection of K8-S\textsuperscript{431}E alone was also sufficient to induce perinuclear organization of endogenous keratin 8 and 18 (Fig. 6D). Thus phosphorylation of K8 at Ser\textsuperscript{431} is required and sufficient for perinuclear keratin reorganization in human pancreatic and gastric cancer cells.

**Keratins regulate the motility of epithelial tumor cells**

Previously, we have shown that SPC reorganizes the keratin cytoskeleton and facilitates migration of tumor cells through size limited pores suggesting a link between keratin organization and tumor cell migration (Beil et al., 2003). To further determine the precise role of keratin in tumor cell migration, we targeted K8 by small interfering RNA oligonucleotides. Depletion of K8 by siRNA was tested by qRT-PCR, immunocytochemistry and Western blot analysis. K8-mRNA transcripts were reduced by 80% 24 hours after transfection (Fig. 7A and S5). A maximum effect on K8 protein expression was observed after 72 hours of incubation with the specific siRNA (Fig. 7B). Depletion of K8 or K18 resulted in a significantly enhanced random migration of tumor cells (siK8 = 0.31 µm/min vs. siControl = 0.18 µm/min; Fig. 7C and D). Thus, under basal conditions the established keratin cytoskeleton obviously restricts tumor cell motility.

SPC stimulates random motility of Panc-1 cells (Fig. 7C and S6A). This increase in motility corresponds to the changes in keratin organization in response to SPC (Fig. S6B) and it was prevented when cells were incubated with the MEK-inhibitor U0126 (Fig. 7E). This was not only the case for random motility but also for tumor cell migration through size limited pores in Boyden chamber assays (Fig. 7F). Interestingly, SPC stimulated random tumor cell migration to a similar degree as keratin depletion, was not able to further enhance random motility of K8-depleted cells (Fig. 7C). Thus, SPC-induced cell migration requires ERK activity and SPC affects tumor cell migration by a mechanism that requires MEK-mediated phosphorylation.

**Phosphorylation of K8 at Ser\textsuperscript{431} leads to enhanced tumor cell migration**

Since perinuclear keratin reorganization correlates with increased tumor cell motility, we next examined the effect of K8-S\textsuperscript{431} phosphorylation on random migration of tumor cells. Pancreatic cancer cells expressing wild type K8/K18 move with a speed of about 0.19
µm/min which corresponds to the speed of untransfected control cells (0.21 µm/min). K8-S^{431}E/K18-wt expressing cells moved twice as fast as wild type cells (0.39 µm/min) and as fast as cells expressing K8-wt/K18-wt in the presence of SPC (0.36 µm/min; Fig. 7G). In addition, the migration speed of K8-S^{431}E/K18-wt expressing cells was comparable to that of K8-depleted pancreatic cancer cells using specific siRNA (0.31 µm/min). Pancreatic cancer cells expressing the K8-S^{431}A/K18-wt mutant exhibited the same migratory behavior as cells expressing wild type K8/18 (0.21 µm/min). SPC failed to enhance the migration velocity of cells expressing K8-S^{431}A/K18-wt (0.24 µm/min; Fig. 7G). This indicates that phosphorylation of K8 at Ser^{431} plays a crucial role for both SPC-induced keratin organization and SPC-induced release of the keratin-mediated inhibition of human tumor cell migration. It has been reported that K8/K18 modulates the distribution of focal adhesions in simple epithelial cells (Bordeleau et al., 2010). Our data (Fig. S7) show that upon expression of K8-S^{431}E there was a significant increase in the number but not the size of focal adhesions. The focal adhesions aligned at the margin of the cells. Thus, keratin phosphorylation affects focal adhesion formation in pancreatic cancer cells and could thereby contribute to the promigratory effect observed. These data provide a direct link between keratin organization, ERK-mediated keratin phosphorylation and tumor cell migration. They further demonstrate that K8-S^{431} phosphorylation resembles a knockdown of keratins by releasing the restrictive function of keratins on tumor cell migration.

Discussion

Sphingosylphosphorylcholine (SPC) is a naturally occurring, bioactive lipid which acts as an intracellular and extracellular signaling molecule in numerous biological processes including proliferation (Seufferlein and Rozengurt, 1995), cell migration (Boguslawski et al., 2000), wound healing (Wakita et al., 1998) and differentiation (Kleger et al., 2007). Previously we established that SPC is one of the few physiological compounds that is able to reorganize the keratin cytoskeleton. SPC induced reorganization of keratin from a widespread, ramified network to a strictly perinuclear, ring-like structure in human epithelial tumor cells (Beil et al., 2003). This reorganization led to an increase in the elasticity of the tumor cells, facilitated migration of cells through size limited pores and was accompanied by keratin phosphorylation at K8-S^{431} and K18-S^{52}.
Members of the MAPK family have previously been shown to mediate phosphorylation of keratins (Omary et al., 2009; Omary et al., 2006, Wöll et al., 2007). Posttranslational modifications are major regulators of keratin function (Ku et al., 1999; Magin et al., 2007; Omary et al., 1998; Toivola et al., 2004). In particular, serine phosphorylation of keratins plays a major role in various cellular events including apoptosis and mechanical or non-mechanical stress (Felder et al., 2008; Jeon et al., 2006; Liao et al., 1997; Liao et al., 1995a; Ridge et al., 2005). Serine hyperphosphorylation of keratins, e.g. by treatment of cells with okadaic acid, leads to disruption of the filament network (Ku and Omary, 1994; Ku et al., 1999; Strnad et al., 2002). Additionally, soluble keratins often exhibit increased serine phosphorylation (Omary et al., 2006; Stossel, 1993). A closer examination of the physiological role of keratin phosphorylation has so far been hampered by the fact that there are only few compounds that physiologically stimulate keratin phosphorylation. Our data point to a central role of K8-S431 in SPC-induced keratin reorganization.

Our data suggested a close relationship between K8-S431 phosphorylation and keratin reorganization in epithelial tumor cells. Phosphorylation of K18 at Ser52 has been described in response to cellular stressors such as heat, viral infection or during mitosis (Ku and Omary, 1994; Ku et al., 1999; Liao et al., 1995b). Furthermore, K18-S52 phosphorylation seems to protect hepatocytes from toxic injury (Omary et al., 2006) and could therefore act as a protective signal in response to injury or stress (Omary et al., 2009; Toivola et al., 2010). SPC-induced phosphorylation of K18 at Ser52 was independent of MEK-ERK activity in Panc-1 and AGS cells. SPC can induce multiple signaling pathways in different cell lines. However, SPC failed to induce significant activation of other pivotal signaling pathways such as the PI3-K/AKT, the PKC/PKD and the PKM2 pathway in our model cancer cell lines. These data underline the importance of ERK-dependent keratin phosphorylation for keratin reorganization in these cell lines. In line with the data described above, experiments using phosphomimetic mutants of K8-S431 and K18-S52 showed that phosphorylation of K18 at Ser52 was neither required nor sufficient for the SPC-induced keratin reorganization. In striking contrast, phosphorylation of K8 at Ser431 was required and sufficient to trigger perinuclear keratin reorganization.

Our previous data suggested that SPC-induced keratin reorganization and cell migration are linked (Beil et al., 2003). In addition, enhanced migration often corresponds to increased activity of the ERK signaling cascade (Huang et al., 2004). Thus, we investi-
gated the role of SPC-induced, ERK-dependent K8 phosphorylation/reorganization in cell migration, both, in the absence and presence of SPC. Inhibition of MEK blocked K8-S\textsuperscript{431} phosphorylation, keratin reorganization and tumor cell migration. Interestingly, depletion of K8 in epithelial tumor cells also resulted in a marked increase in tumor cell motility compared to cells transfected with a scrambled siRNA construct. These data are in line with observations in wound healing where knockdown of keratin 8 with siRNA resulted in accelerated wound closure in vimentin-positive HeLa and Panc-1 cells (Long et al., 2006). Notably, compared to control cells, scratch wound edges were irregular after K8 depletion and frequently contained cells that were migrating individually in both cell lines (Long et al., 2006). This behavior of K8 knockdown cells is comparable with our single-cell based experiments and indicates that the basal organization of the keratin cytoskeleton in simple epithelial tumor cells serves to restrict cellular migration. Interestingly, SPC failed to further increase migration velocity of tumor cells depleted of K8. This shows that keratin organization is crucial for the effect of SPC on tumor cell migration. In addition, our data clearly show that ERK-induced phosphorylation of K8 at Ser\textsuperscript{431} is sufficient to stimulate tumor cell migration to a similar degree as SPC treatment. Accordingly, phosphorylation at K8-S\textsuperscript{431} was required for SPC-induced tumor cell migration. The migratory behavior of tumor cells depleted of K8 was similar to that of cells expressing the phosphomimetic K8-S\textsuperscript{431}E mutant. This shows that ERK-mediated K8-S\textsuperscript{431} phosphorylation functionally mimics K8 depletion of tumor cells and abolishes the otherwise restrictive function of the keratin cytoskeleton on tumor cell migration. The fact that this effect can be so clearly demonstrated using SPC, but has so far not been described for other activators of the ERK cascade such as EGF/EGFR may lie in the fact that SPC by acting via a GPCR induces only few signaling pathways in human epithelial tumor cells, in particular an activation of the ERK cascade (our own unpublished observations). Upon induction of multiple pathways there are likely to be other modifications of keratins that attenuate or modulate the effect of ERK activation with respect to keratin reorganization.

Our data have implications for tumor biology. The ability of tumor cells to migrate is pivotal for tumor progression and metastasis (Chambers et al., 2002). Our data show that ERK-dependent phosphorylation of K8-S\textsuperscript{431} leads to increased tumor cell migration due to reorganization of the keratin cytoskeleton and this is likely to contribute to the metastatic properties of these cells. Thus, we provide a novel mechanism how ERKs can re-
gulate tumor cell migration and identify keratin phosphorylation as an interesting novel therapeutic target to prevent invasion and potentially metastasis.
Materials and Methods

Materials
The MEK Inhibitor U0126 was obtained from Promega (Fitchburg, WI, USA). PD98059 and SPC were from Calbiochem (Merck Chemicals, Nottingham; UK), TGF-β from BD Biosciences (San Jose, CA, USA). Antibodies directed against p44/p42 and phospho-p44/42 are from Cell Signaling (Danvers, MA, USA), antibodies detecting Pan-Cytokeratin (Pan-CK, clone KL1) are from Immunotech (Praha, Czech Republic). The phosphospecific antibodies detecting K18-S\(^{52}\) (3055) and K8-S\(^{431}\) (5B3) have been described previously (Ku et al., 1997; Liao et al., 1995b). Antibodies directed against Akt, phospho-Akt (Ser 473), phospho-PKD (Ser744/748) and phospho-PKM2 (Tyr105) were obtained from Cell Signalling (Danvers, MA, USA). The mouse monoclonal Ab to PKM2 was purchased from Abcam (Cambridge, UK). The antibodies detecting PKD2 were purchased from Calbiochem (Merck Chemicals, Nottingham; UK). Phorbol-12-myristate-13-acetate (PMA) was obtained from Calbiochem (Merck Chemicals, Nottingham; UK). IGF1 was from Peprotech (Hamburg, Germany).

Cell culture and reagents
Panc-1 human pancreatic cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA). AGS cells were a gift of M. Hoecker (Charite, Berlin, Germany). This is a human gastric cancer cell line that also exclusively expresses keratins, but no vimentin. Cells were maintained in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (PAA, Pasching, Austria) in a humidified atmosphere and 5% carbon dioxide, 95% air at 37°C and passaged every 2-3 days. Cells were incubated in serum-free DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) 18 hours according to the experiment.

Cell transfection
For Immunofluorescence microscopy and live-time imagine, cells were either transfected with Fugene (Roche, Basel, Switzerland) or Metafectene (Biontex, Martinsried, Germany). For Migration assays transfection was performed with Nucleofector (Amaxa, Cologne, Germany) using Kit R and program X-005, siRNA was transfected with RNAiFect (Qiagen).
**Immunofluorescence microscopy**

Pan-CK antibody and the phosphospecific antibody detecting K8-S$^{431}$ (5B3) were used for immunofluorescence microscopy. After the indicated stimulation, cells were fixed with 4% formaldehyde for 10 minutes. Antibodies were added overnight in PBS supplemented with 0.5% TritonX-100 and 0.2% Gelatin (Sigma, St. Louis, MO, USA) at 4°C. Cells were then incubated with Alexa 488, 568 or 647 coupled to secondary antibodies (Invitrogen, Carlsbad, CA, USA). Finally, slides were embedded in GelTol Aqueous Mounting Medium (Immunotech, Praha, Czech Republic). Imaging was performed with confocal laser scanning microscope LSM510 Meta (Carl Zeiss, Jena, Germany) equipped with a 63x/1.4 oil objective using the indicated filters or a Keyence BZ-8000 fluorescence-microscope. Images show representative cells from at least three independent experiments.

**Cell migration through size-limited pores**

Panc-1 cell migration was examined using a modified 48-well Boyden chamber (Nucleopore, Neuro Probe, Gaithersburg, MD, USA) and collagen-coated polycarbonate membranes with a pore diameter of 12 µm (Nucleopore). Panc-1 cells ($2 \times 10^5$ cells ml$^{-1}$) in DMEM were allowed to migrate towards a gradient of the indicated agents for a total of 4 hours in a humidified incubator (37°C; 5% carbon dioxide). Adherent cells on the filter membrane were fixed in 99% ethanol for 10 minutes and stained using Giemsa dye. For a quantitative assessment of migrated cells, from three different wells in each case 5 high-power fields (15 in total) were counted. The data shown represent the percentage of migrated cells, compared with the unstimulated control.

**Migration assays with time-lapse microscopy**

Panc-1 cells were seeded on fibronectin (Roche Diagnostics, Penzberg, Germany) covered glass-slides 3 hours before starting of migration assay. Glass-slides were packed together with serum free DMEM containing 1% Pen/Strep into a sample-sandwich and sealed with wax. The sample-sandwich was kept at 37°C and time-lapse was run for 16 hours. K8-wt/K18-wt, K8-SE/K18-wt and K8-SA/K18-wt expressing cells were transfected 24 hours before the start of migration assays. Time-lapse photos were analyzed using ImageJ (http://rsb.info.nih.gov/ij/java 1.3.1_13).
Western blot analysis and extraction of keratins

For keratin extraction, serum-starved cultures of Panc-1 and AGS cells were treated with factors as indicated and lysed at 4°C in 20 mM Tris-HCl at pH 7.4, 0.6 M potassium chloride, 1% Triton X-100 and 1 mM PMSF (Triton high-salt buffer). Lysates were incubated for 20 minutes on ice and cleared by centrifugation at 10,000 g for 20 minutes at 4°C. The resulting pellet was subsequently resuspended in the same buffer, incubated for a further 20 minutes on ice and again subjected to centrifugation at 10,000 g for 20 minutes at 4°C. The pellet of insoluble proteins was resuspended in 5 volumes of 8 M urea and the same volume of 5x SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer was added to the solution. Samples were then separated by SDS–PAGE.

For Western blot analysis, serum starved cultures of Panc-1 and AGS cells were treated as indicated and lysed at 4°C in NP-40 lysis buffer [150 mM NaCl, 20 mM Tris-HCl, 10% glycerol, 1% NP-40, Na₄O₇P₂ (100 mM), NaVO₄ (100 mM), NaF (1 M), aprotinin (10 µg/ml), leupeptin (100 µg/ml), pepstatin (0.7 µg/ml)]. Lysates were incubated for 10 minutes at 4°C and subjected to centrifugation at 14,000 g for 10 minutes at 4°C. Supernatants of the samples were resuspended in 5x SDS-PAGE sample buffer and separated by SDS-PAGE.

Quantification of keratin morphology

Panc-1 and AGS cells were transfected as indicated and keratin morphology was quantified by determining the percentage of cells with a predominant perinuclear or ramified keratin organization using fluorescence microscopy. Keratin organization was classified as predominantly perinuclear when more than 70% of keratin as assessed by visual judgment, was organized around the nucleus and consequently the cytoplasm contained less keratin filaments. The formation of a strict ring structure was frequently observed, but not required for the classification as perinuclear. Keratin organization was classified as predominantly cytoplasmic when more than 70% of keratin as assessed by visual judgement was organized in the cytoplasm and consequently the cytoplasm contained more keratin filaments than the perinuclear region. Quantification was performed in a blinded fashion in at least 3 independent experiments with 50 to 200 cells per experiment.
Quantification of the fluorescence intensity distribution

For quantification of keratin distribution visualized by indirect immunofluorescence or CFP-tagged keratins as described in the Figure Legends ortho-max projections of confocal image sections from top to bottom of each cell were analyzed. Cells were imaged using a SP5 confocal microscope (Leica) with similar settings. Three linear ROIs of equal length were placed in perinuclear and cytoplasmic regions of each cell (LAS AF Lite Software, Leica). The normalized intensity profile for all ROI was integrated by calculating area under the curve and the mean intensity ratio of perinuclear/cytoplasmic ROIs for each cell was used an indicator for cytokeratin distribution under different conditions. Height of cells (Z-Volume) was calculated from confocal image stacks. Calculations and statistical analysis was performed using GraphPad Prism version 5.00.

Reverse transcriptase and PCR

mRNA was prepared from either Panc-1 or AGS cells and semi-quantitative RT-PCR analysis was conducted with specific primers for SPC receptors as described before (Kleger et al., 2007).

Real-time PCR

Keratin 8 and 18 mRNA levels were measured using the LightCycler System (Roche Diagnostics, Mannheim, Germany) or the ICyclerIQ system from Biorad.

Primers used in real time-PCR were as follows: h-Keratin 18: QuantiTect Primer Assay Hs_KRT18_2_SG. h-Keratin 8: 5’-GCCGTGGTTGTGAAGAA-3’ and 5’-CTGGTCCAGTGTACCTACCT-3’. h-HMBS: 5’-CCCTGGAGAAGAATGAAGTGGA-3’ and 5’-TGGGTGAAAGACAACAGCATC-3.

siRNA

For keratin 8 knock-down, the siRNA probe: GGGUGACCCAGAAGUCCUA labeled with 3’-fluorescienc or 3’-AlexaFluor488 was used. As a control, an AllStar siRNA labeled with AlexaFluor546 (Qiagen, Hilden, Germany) was used. To deplete keratin 18, we used a mixture of two different siRNA constructs: Stealth RNAi KRT18-HSS142770 (CatNo: 5194538, Invitrogen) and Hs-KRT18-3 CK (5’-ccgccgtagtggatgca-3’, 5’-gccggauaguggcaatt) (Qiagen). AllStar siRNA labelled with AlexaFluor546 (Qiagen) was used as a control. For ERK1/2 knockdown p44/42MAPK (ERK1/2) siRNA from Cell
Signalling was used. Panc1 cells were transfected using Hyperfect (Qiagen) according to manufacturer’s instructions.

Site-directed mutagenesis
To obtain amino acid exchange of keratin 8-Ser\(^{431}\) and keratin 18-Ser\(^{52}\), site-directed mutagenesis with pK8-eCFP and pK18-eYFP (R. Leube, Universitätsklinikum Aachen, Germany; Wöll et al., 2005) as matrices were performed using QuikChange\(®\)XL-sdm-Kit (Stratagene, La Jolla, CA, USA) according to instruction manual. For exchange of K8-S\(^{431}\) and K8-S\(^{52}\) with Alanine (A) or Glutamic acid (E) primers as follows were used:

K8-S\(^{431}\) → A: TATGGGGGCCTCCAGCCCCC-GGCCTCA; K8-S\(^{431}\) → E: CTATGGGGGCCTCACAGAACCCGGGCTCACTACAG; K18-S\(^{52}\) → A: ATCTCCGTGTCGACCAGCTTCAGGANC; K18-S\(^{52}\) → E: GTGTCCCCGCTCCACCGAAAAGGGCGGCATG.

Supplementary methods

Random Migration
Serum-starved Panc-1 cells were subjected to a random migration on fibronectin and imaged by the time-lapse video microscopy. Glass bottom culture dishes (MakTek Corporation) were coated with 50 µg/ml of fibronectin (Roche). Cells were allowed to spread for 3 h on the fibronectin coated dishes in DMEM with 1% FCS. Imaging was performed using a BZ-8000 Keyence microscope. During imaging cells were kept at 37°C in an atmosphere containing 5% CO\(_2\). A motion picture (AVI format) was created from time-lapse images using the BZ-Analyser software (Keyence Corporation). Cell movement was analysed using tracking routines implemented in the ImageJ software. Three independent experiments were done for each condition.

Isolation and Analysis of Keratin Fractions
Keratins were isolated from three cellular fractions. The cytosolic fraction was obtained after disrupting cells by centrifugation at 100,000g for 90 min in Buffer A [PBS 10 mM EDTA, Protease/Phosphatase Inhibitor Cocktail (Roche)]. The pellet was then solubilised using 1% NP-40 in buffer A (30 min at 4°C) followed by centrifugation (16,000 g, 15 min, 4°C) and collecting the NP-40 fraction. The remaining cytoskeletal fraction was solubilised in 50 mM Tris-HCl, pH 7.4, 2 mM EDTA / 9.5 M urea.
Acknowledgement

We thank R. Leube (Aachen) for providing the keratin vectors pK8-eCFP and pK18-eYFP, C. Ruhland and U. Mayr-Beyrle for expert technical assistance. This work was supported by the Deutsche Krebshilfe Grant 107344 to T.S., and National Institutes of Health grant DK47918 to M.B.O.
References


Figure legends

Figure 1: SPC-induced activation of p44/42 phosphorylation
A: Panc-1 and AGS were incubated with 12.5 µM SPC for the indicated time points. B: Cells were treated with various concentrations of SPC as indicated for 15 min (Panc-1) or 30 min (AGS). C: Panc-1 and AGS were preincubated with PD98059 using the concentrations indicated and subsequently treated with 12.5 µM SPC for 15 min (Panc-1) or 30 min (AGS). D: Panc-1 and AGS cells were preincubated with the U0126 as indicated and subsequently treated with 12.5 µM SPC for 15 min (Panc-1) or 30 min (AGS). Immunoblots were performed using antibodies against p44/42 and phosphorylated p44/42 (Ph-p44/42).

Figure 2: Keratin reorganization in epithelial tumor cells after SPC treatment
A: Panc-1 and AGS cells were transfected with eCFP-tagged K8 / eYFP-tagged K18 or left untransfected and were subsequently incubated with 15 µM SPC for 45 min. Endogenous keratins were stained with pan-CK antibody, followed by Alexa488 staining. Images were taken using a confocal microscope and keratin was detected within the 488 channel (green emission). Representative pictures show reorganization of endogenous or ectopic keratin filaments to a perinuclear, ring-like structure upon SPC incubation. Size bar = 10 µm. B: Quantification of cytokeratin organization. Cytokeratin organization was quantified in cells treated with/without 12.5 µM SPC for 1 h, as described in methods. Images represent ortho-max projections of confocal image sections with three linear ROIs in the perinuclear and the cytoplasmic region, respectively. Left Graph show intensity ratio of perinuclear/cytoplasmic ROIs. Cells treated with SPC exhibit an increase in fluorescence intensity in the perinuclear area compared to not treated demonstrating a marked difference in cytokeratin redistribution upon phosphorylation that can be quantified. Right graph show height of cells (Z-Volume) calculated from confocal image stacks (0, 01 to 0, 05 *; 0,001 to 0, 01 **; ***; p<0,001 ****).

Figure 3: Role of p44/42 activation in SPC-induced keratin reorganization
A-B: Cells were plated on cover slips and subsequently transfected with the respective plasmids for 48h. Panc-1 (A) and AGS (B) cells were incubated with either 15 µM
PD98059 (only Panc-1) or 10 µM U0126 for 1 h followed by 45 min incubation with 15µM SPC. Photographs of representative cells (two different magnifications in A) show endogenous (end.) stained with a pan-CK antibody, followed by Alexa488 staining or transfected eCFP-K8-wt / eYFP-K18-wt keratin (ectopic = ect.). Images were taken using a confocal microscope and keratin was detected within the 488 channel. Size bars = 10 µm. A and B, bar graphs: All cells on the cover slip or all transfected cells, respectively, were counted (between 50 and 200 cells per coverslip) and the ramified versus perinuclear phenotype of keratin was assessed by a person blinded for the specific condition. Data are expressed as the percentage of cells exhibiting a ramified or a perinuclear keratin phenotype and are the means +/- SEM of 3-10 independent experiments per condition.

Figure 4: p44/42 mediates SPC-induced keratin phosphorylation

A-B: Keratins were extracted from Panc-1 and AGS cells after treatment of 15 µM SPC and/or 15 µM PD98059 and 10 µM U0126. Keratin phosphorylation was analyzed by Western blot with antibodies against phospho-K8-S431 (Ph-K8-S431; A) or phospho-K18-S52 (Ph-K18-S52; B). Representative immunoblots are shown. Graphs display quantifications of luminescence. Error bars indicate the SEM of at least 4 independent experiments. Significant differences were calculated using Student T-test [(*) represents a significant difference between marked columns and not treated control; (#) represents a significant difference between marked columns and 60 min SPC treatment; p < 0.05 (*, #); p < 0.01 (**, ##); p < 0.001 (***, ###)].

C: Panc-1 and AGS cells were treated with 15 µM SPC for 45 min and keratin immunocytochemistry was performed using pan-CK and phospho-K8-S431 (Ph-K8-S431) antibodies, followed by Alexa488 staining. Photographs show representative cells at two different magnifications. Size bars = 10 µm.

D: Panc-1 and AGS cells were treated with 15 µM PD98059 or 10µM U0126 for 1h followed by incubation with 15 µM SPC for 45 min. Immunocytochemistry was performed using a phosphospecific antibody against K8-S431 (Ph-K8-S431). Graph depicts quantification of Ph-K8-S431 positive cells compared to all stained cells [(*) represents significant difference between marked columns and not treated control; (#) represents significant difference between marked columns and 45 minutes SPC treatment; p < 0.01 (**, ##); p < 0.001 (***, ###)].

E: Left panel: p44/42 was depleted in Panc-1 cells using specific siRNA as described in Materials and Methods. p44/42 depletion was confirmed by Western blotting. Right panel: p44/42 was depleted in Panc-1 cells followed by SPC
treatment (20 min; 12.5μM SPC) and keratin immunocytochemistry was performed using a phosphospecific antibody directed against phospho-K8-S^{431}(p-K8-S^{431}). Intense p-K8-S^{431} immunoreactivity was detected exclusively upon incubation of cells with SPC, but not in unstimulated, control cells or cells in which p44/42 were depleted. p-K8-S^{431} immunoreactivity was predominantly detectable in reorganized, perinuclear keratin filaments indicating that K8 phosphorylation strictly correlates with keratin reorganization. Photographs show representative cells at two different magnifications. Size bars = 10μm. Graph depicts quantification of p-K8-S^{431} positive cells compared with all cells. Error bars indicate SEM of 3 independent experiments. Significant difference was calculated using the Student T-test (p < 0.05 (*)). (M) indicates merged image.

Figure 5: Effect of modifying Ser431 in K8 and Ser52 in K18 on keratin organization in pancreatic cancer cells
A-E: Panc-1 cells were transfected with K8-wt/K18-wt (A), K8-SA/K18-SA (B), K8-SE/K18-SE (C), K8-wt/K18-SE (D) or K8-wt/K18-SA (E) and treated with 15 μM SPC and/or 10 μM U0126 as indicated (eCFP-tagged K8; eYFP-tagged-K18). Images were taken using a confocal microscope and keratin was detected within the 488 channel. Graphs display quantification of cells with perinuclear or ramified keratin compared to number of transfected cells.

Figure 6: K8-S^{431} phosphorylation is sufficient to trigger perinuclear keratin organization in Panc-1 cells
A-C: Panc-1 cells were transfected with K8-SE/K18-wt (A), K8-SA/K18-wt (B) or K8-SE/K18-SA (C) and treated with 15 μM SPC and/or 10 μM U0126 as indicated (eCFP-tagged K8; eYFP-tagged-K18). Keratin was detected within the 488 channel. Images show representative cells. Graphs display quantification of cells with perinuclear or ramified keratin compared to the total number of transfected cells. D: Panc-1 cells were transfected with either eCFP-K8-wt or eYFP-K8-SE and stained with pan-CK antibody, followed by labelling with anti-mouse Alexa647 Ab (Pan-CK). Images were taken using a confocal microscope and keratin was detected within two channels. E: Quantification of cytokeratin organization. Cytokeratin organization was quantified in cells expressing wild type cytokeratin 8-CFP or cytokeratin 8 SE-CFP-mutant, as described in methods. Images represent ortho-max projections of confocal image sections with three linear ROIs in the perinuclear and the cytoplasmic region, respectively. Left graph show
intensity ratio of perinuclear/cytoplasmic ROIs. Cells overexpressing the phosphomimetic keratin 8 mutant (CFP-K8 se) exhibit an increase in fluorescence intensity in the perinuclear area compared to cells overexpressing wild type keratin 8, respectively, demonstrating a marked difference in cytokeratin redistribution upon phosphorylation that can be quantified. Right graph show height of cells (Z-Volume) calculated from confocal image stacks (0,01 to 0,05 *; 0,001 to 0,01 **; p<0,001 ***; p<0001 ****).

Figure 7: Effect of a keratin depletion on basal and SPC-induced random migration of pancreatic cancer cells

A: K8 and/or K18 were depleted in Panc-1 cells using specific siRNAs (siK8, siK18). Relative K8 mRNA (upper panel) and K18 mRNA levels (lower panel) were determined in Panc-1 cells by qRT-PCR using the iCyclerIQ system from Biorad. Graphs depict mRNA expression in percent, as compared to control. Figures shows representative data obtained in triplets of at least 3 independent experiments. B: Upper panel: Panc-1 cells were transfected with specific K8-siRNA (siK8) and/or K18-siRNA (siK18). After 72 hours keratins were extracted and expression of keratins analysed by Western blotting using a pan-CK antibody. Lower panel: Densitometric evaluation of keratin protein levels. Data represent the means +/- SEM of three independent experiments. C: Panc-1 cells were transfected with scrambled siRNA (siCon) or K8-siRNA. After 48 h cells were incubated with 10 µM SPC (+) or solvent (-) and subjected to a random migration that was analysed by time-lapse video microscopy. Cells were tracked and the velocity was calculated using the ImageJ program. Data represent the fold increase in migration above control and are the means +/- SEM of three independent experiments. D. Panc-1 cells were transfected with scrambled siRNA (siCon) or K18-siRNA. 24h after knockdown cells were incubated with solvent or 10 µM SPC, subjected to random migration. Migration was analysed as described above. Data represent the fold increase in migration above control and are the means +/- SEM of three independent experiments. E: Panc-1 cells were incubated with 10 µM U0126, 10 µM SPC and 5 ng/ml TGF-β as indicated. Random migration was determined via time-lapse video microscopy as described above. Data represent the fold increase in migration above control and are the means +/- SEM of four independent experiments. F. Panc-1 cells were incubated with 10 µM U0126, 10 µM SPC and 5 ng/ml TGF-β as indicated. TGF-β was used as a positive con-
trol (Rahimi and Leof, 2007). Migration through size limited pores was determined using a Boyden-chamber assay as described before (Beil et al., 2003). Data represent the fold increase in migration above control and are the means +/- SEM of three independent experiments. G. Panc-1 cells were transfected with K8-wt/K18-wt, K8-SE/K18-wt or K8-SA/K18-wt and subsequently incubated with 10 µM SPC (+). Random migration was determined as above. Data represent the fold increase in migration above control and are the means +/- SEM of four independent experiments.

In all experiments described in 7C-G significant differences were tested using the Student T-test (p<0.01 (**); p<0.001(***)).

Supplementary Figure Legends

Figure S1A: Subcellular distribution of overexpressed and endogenous K8 and endogenous K18. B: Expression of SPC receptors in Panc-1 and AGS cells: mRNA was isolated from Panc-1 and AGS cells and RT-PCR was performed using primers for sphingosine-1-phosphate receptors (S1P) 1-5, GPR4 and OGR1 (positive control (pos) =lung/liver).

Figure S2: A: To assess the relationship between keratin phosphorylation and its solubility, we analyzed cytosolic, NP-40-solubilized, and urea-solubilized cell fractions that were sequentially separated from cells transfected with K8-wt (CFP-K8-wt), non-phosphorylatable (CFP-K8-SA) or phospho-mimetic keratin 8 (CFP-K8-SE). The cytosolic fraction represents the soluble cellular fraction isolated after cell disruption in the absence of any detergent by centrifugation at 100,000g. The remaining post-cytosolic pellet is then solubilized in NP-40 which, with regard to K8/18, would be expected to solubilize some of the membrane and "loosely associated" cytoskeletal fraction (Chou et al., 1994). The post-NP-40 pellet (cytoskeletal fraction) is then solubilized in 9.5 M urea. As shown, each cellular pool contains K8/18. In addition, there is an increase in the K8 cytosolic fraction of cells transfected with phosphomimetic K8 compared to cell transfected with wild type or non-phosphorylatable K8. Graph represents densitometric quantification of CFP-K8 protein level in cytosolic/soluble fraction. B: SPC exclusively activates the p44/p42 ERKs. Panc-1 cells were treated with 12.5 µM SPC. At the indicated time points either a keratin or a cytoplasmic extract was prepared. Equal amounts of the keratin fractions or 50 µg cytoplasmic extract were separated by SDS-PAGE, blotted and incubated with antisera detecting either the phosphorylated (ph-) or the total.
amount of the proteins indicated on the left. ph+: Panc-1 cells were treated either with 200 nm PMA (for the PKD2 blots) or with 10 µM IGF1 (for the AKT blots) for 10 min to achieve maximum phosphorylation of PKD2 and Akt, respectively. 50 µg cytoplasmic extract was loaded as a positive control for the phosphospecific antibodies. * marks the band corresponding to ph-Akt.

Figure S3: Effect of modifying Ser431 in K8 and Ser52 in K18 on keratin organization in AGS cells. A-E: AGS cells were transfected with K8-wt/K18-wt (A), K8-SA/K18-SA (B), K8-SE/K18-SE (C), K8-wt/K18-SE (D) and K8-wt/K18-SA (E) and treated with 15 µM SPC and/or 10 µM U0126 (eCFP-tagged K8; eYFP-tagged-K18). Keratin was detected within the 488 channel using confocal microscope. Images show representative cells. Graphs display the quantification of cells with perinuclear or ramified keratin compared to number of transfected cells.

Figure S4: K8-S431 phosphorylation is sufficient to trigger perinuclear keratin organization in AGS cells
A-C: AGS cells were transfected with K8-SE/K18-wt (A), K8-SA/K18-wt (B) and K8-SE/K18-SA (C) treated with 15 µM SPC and/or 10 µM U0126, (eCFP-tagged K8; eYFP-tagged-K18). Keratin was detected within the 488 channel. Images show representative cells. Graphs display quantification of cells with perinuclear or ramified keratin compared to number of transfected cells.

Figure S5: Keratin 8 knock-down. A: Panc-1 cells were transfected with K8-siRNA and mRNA was isolated at the times indicated. Relative K8 mRNA levels were measured with real-time PCR using a LightCycler system. The graph depicts K8-mRNA expression in percent as compared to control. Data are the means +/- SEM of 3 independent experiments.

Figure S6: A: Random migration of SPC-treated Panc-1 cells. Panc-1 cells were incubated with SPC (10 µM) or solvent, subjected to random migration and imaged by time-lapse video microscopy for 45 min, 2h, 6h, 12h or 16h as indicated. Cells were tracked and the velocity was calculated using the Image J program. Graphs show the increase in cell velocity [µm/min] in SPC treated cells compared to solvent treated cells. Error bars indicate the means +/- SEM of 3 independent experiments. p < 0.05 is indi-
B: Keratin reorganization in epithelial tumour cells upon SPC treatment. Panc-1 cells were incubated with 10 μM SPC for different times as indicated. Endogenous keratin was stained using a pan-CK antibody. Representative images show the reorganization of endogenous keratin filaments to a perinuclear, ring-like structure upon SPC incubation in a time dependent manner. Size bar = 10 μm. Graphs display quantification of cells with perinuclear or ramified keratin (C, D). p < 0.05 is indicated by *.

Figure S7: A: The effect of Keratin 8 phosphorylation on actin and focal adhesion organisation. To assess the relationship between keratin phosphorylation and actin / focal adhesion organisation, Panc 1 cells were transfected with wild type keratin 8 (CFP-K8wt) or phospho-mimetic keratin 8 mutant (CFP-K8SE). Serum-starved cells were then fixed and stained with Alexa Fluor 568 labelled phalloidin to show actin filaments, and paxillin as marker for focal adhesions. Significant changes in pattern of Actin organisation related to phosphorylation of keratin were not observed. Panc-1 cell show variable actin organisational pattern independent of keratin phosphorylation. This variability was observed in cells overexpressing wild type as well in CFP-K8SE overexpressing cells. B: Size and number of paxillin positive focal adhesions were quantified using the Image J software. Twenty cells were analysed per experiment in total of six independent experiments. Average size and number of focal adhesions were normalised to the size of the cell. Calculations show that the number of focal adhesions formed in cells over-expressing CFP-K8SE is significantly (* P <0.05) higher compared to cells expressing CFP-K8wt. The size of the focal adhesions remained the same in both conditions. Size bars = 10μm.
Fig. 1

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Fig. 2

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Intensity Ratio perinuclear/cytoplasmic

Z-Volume [μm]

- SPC / pan-K

+ SPC / pan-K

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E.

**Fig. 4E**

- **siCon** vs **siERK**
  - ERK
  - Actin

**p-K8 S431**
- **- SPC**
- **+ SPC**

**% of all cells**
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Heterozygous K8wt/K18wt cells showed a predominance of perinuclear alpha-actinin staining, while heterozygous K8SA/K18SA cells displayed a mixture of perinuclear and ramified staining. Heterozygous K8SE/K18SE cells exhibited a balance between perinuclear and ramified staining, with a slight shift towards ramified staining. Heterozygous K8wt/K18SE cells showed a significant increase in ramified staining, compared to perinuclear staining. Heterozygous K8wt/K18SA cells displayed a similar pattern as K8wt/K18SE, with a slight increase in perinuclear staining.

Fig. 5
E. CFP-K8 wt

Fig. 6
Fig. 7