A new model for regulation of sphingosine kinase 1 translocation to the plasma membrane in breast cancer cells

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ABBREVIATIONS: AUC, area under the curve; CTD, C-terminal domain; ERK, extracellular signal regulated protein kinase; FIPI, N-[2-[4-(2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]ethy]-5-fluoro-1H-indole-2-carboxamide hydrochloride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, Green fluorescent protein; LBL-1, lipid-binding loop 1; NTD, N-terminal domain; PA, phosphatidic acid; PLD, phospholipase D; PM, plasma membrane; PMA, phorbol 12-myristate 13-acetate; PP2A, protein phosphatase 2; PS, phosphatidylserine; R-loop, regulatory loop; S1P, sphingosine 1-phosphate; SK1, sphingosine kinase 1; TRAF2, tumour necrosis factor receptor-associated factor 2.
Abstract
The translocation of sphingosine kinase 1 (SK1) to the plasma membrane (PM) is crucial in promoting oncogenesis. We have previously proposed that SK1 exists as both a monomer and dimer in equilibrium, although it is unclear whether these species translocate to the PM via the same or different mechanisms. We therefore investigated the structural determinants involved to better understand how translocation might potentially be targeted for therapeutic intervention. We report here that monomeric wild type (WT) mouse SK1 (GFP-mSK1) translocates to the PM of MCF-7L cells stimulated with carbachol or phorbol myristate acetate (PMA), whereas the dimer translocates to the PM in response to sphingosine 1-phosphate (S1P); thus, the equilibrium between monomer and dimer is sensitive to cellular stimulus. In addition, carbachol and PMA induced translocation of monomeric GFP-mSK1 to lamellipodia, while S1P induced translocation of dimeric GFP-mSK1 to filopodia, suggesting that SK1 regulates different cellular processes dependent on dimerization. GFP-mSK1 mutants designed to modulate dimerization confirmed this difference in localization. Regulation by the C-terminal tail of SK1 was investigated using GFP-mSK1 truncations. Removal of the last five amino acids (PPEEP) prevented translocation of the enzyme to the PM, while removal of the last ten amino acids restored translocation. This suggests that the penultimate five amino acids (SRRGP) function as a translocation brake, which can be released by sequestration of the PPEEP sequence. We propose that these determinants alter the arrangement of N-terminal and C-terminal domains in SK1, leading to unique surfaces that promote differential translocation to the PM.

Introduction
Sphingosine 1-phosphate (S1P) is a bioactive lipid that is formed by the phosphorylation of sphingosine by sphingosine kinase, of which there are two isoforms (SK1 and SK2). SK1 and SK2 are encoded by different genes and have distinct subcellular localisation and biochemical properties (1). S1P is degraded by S1P lyase, to produce (E)-2-hexadecenal and phosphoethanolamine, and by S1P phosphatase, which dephosphorylates S1P to form sphingosine (1). S1P is released from cells via specific transporters in the plasma membrane (PM) and then binds to and stimulates a family of G protein-coupled receptors (GPCRs), the S1P receptors (S1P₁–S1P₅), on cells or can act on intracellular targets, such as histone deacetylase 1/2, to induce cellular responses (1). An important role for S1P in cancer is evident from studies showing that high expression of SK1 and S1P receptors in tumours is linked with poor prognosis in patients (2-4). S1P also promotes transformation, epithelial mesenchymal transition and invasiveness, cancer cell survival, replicative immortality, tumour neovascularisation and aerobic glycolysis—the so-called hallmarks of cancer (1, 5, 6). Therefore, SK1 is a target for therapeutic intervention in cancer. Indeed, oncogenic transformation of NIH3T3 cells is induced by over-expression of SK1 (7) and this involves its translocation from the cytoplasm to the PM, a process that allows access to substrate (sphingosine, Sph). The importance of this process is evident from studies showing that the over-expression of a kinase dead G82D hSK1 mutant fails to induce transformation, thereby demonstrating dependency on the catalytic activity of SK1 and therefore S1P (7).

A key step in the transformation process is the phosphorylation of SK1 by extracellular signal regulated kinase (ERK) on Ser225 in hSK1, which promotes translocation of the enzyme from the cytoplasm to the PM (8, 9). The importance of this is underlined by the fact that the constitutive localisation of SK1 to the PM promotes oncogenic transformation (9). Moreover, studies have demonstrated that SK1 activation and localisation to the PM and subsequent stimulation of the S1P₂ receptor by released S1P (‘inside-out’ signaling) increases transferrin receptor 1 (TFR1) expression (10) and this is critically important in promoting transformation, as evidenced by data showing that a neutralising anti-TFR1 antibody blocks oncogenesis (10).

Dynamically regulated localisation of SK1 to the PM is clearly important for the cellular function of the enzyme. Although the structural basis for this controlled targeting remains incompletely understood at present, the emergence of crystal
structures for SK1 has begun to shed some light on the issue in recent years. The enzyme adopts a bi-domain organisation with a C-terminal domain (CTD) that hosts the lipid substrate binding site and an N-terminal domain (NTD) that binds ATP; the catalytic centre is formed at the interface between these two domains (reviewed in (11)). Sphingosine is bound within a J-shaped cavity (the ‘J-channel’), formed by packing of three extended lipid-binding loop (LBL) structures against a β-sandwich core in the CTD. An exposed hydrophobic patch on the exterior of LBL-1 (L194/F197/L198 in human SK1, hSK1) has been shown to be a key determinant for membrane engagement, including curvature-sensitive binding (12, 13). The same hydrophobic patch on LBL-1 has also been implicated in the binding of SK1 to calcium- and integrin-binding protein (CIB1), a calcium-sensing partner protein that is involved in the translocation of SK1 from the cytoplasm to the membrane (14).

Acidic phospholipids also play a critical role in the membrane localisation of SK1, notably phosphatidylserine (PS) and phosphatidic acid (PA) (15-17). Early studies implicated Thr54 and Asn89 of hSK1 in the interaction of the enzyme with PS (15, 18), but recent attention has focused on a set of basic residues as a candidate interface for acidic phospholipid engagement. The interface was first identified by analysis of hSK1 crystal structures (11). In all five of the currently available structures the protein adopts a dimeric assembly (19-21), consistent with co-immunoprecipitation analysis that showed that SK1 can dimerise (22). The mode of dimerisation generates a pronounced groove at the dimer interface that is aligned parallel with the LBL-1 loops of the two protomers within the assembly. The grooved surface exhibits a pronounced positive electrostatic potential surface, due to contributions from basic residues within the NTD and on the tip of LBL-1, and this is topographically coordinated with the LBL-1 hydrophobic patches to provide a suitable membrane binding interface (11). Recent mutagenesis studies have validated the proposed membrane engagement surface and specifically identified K27, K29 and R186 in hSK1 as residues that are important for binding to PA-enriched target membranes (13).

The Ser225 phosphorylation site for ERK in hSK1 (conserved in mSK1) occupies a prominently solvent-exposed position within an extended regulatory loop (the ‘R-loop’) that packs on the reverse side of the CTD β-sandwich core to LBL-1 and is therefore on the opposite side of the protein to the membrane engagement surface. The mechanism underpinning translocation in response to phosphorylation at this site has not yet been elucidated. However, it may involve induced inter-domain movement that affects the presentation of key basic residues in the NTD relative to LBL-1 in the CTD and thence whether the protein presents a topographically coordinated combination of surface elements or misaligned patches; R-loop phosphorylation might also impact on the capacity of SK1 to adopt a dimeric assembly in principle (11). As an added complexity, there are additional drivers for translocation of SK1 to the PM that are independent of S225 phosphorylation in SK1. For example, Goq has been shown to promote translocation of mSK1 and hSK1 to the PM in HEK293 cells in a manner that is dependent on the generation of the activated Goq subunit but independent of downstream [Ca2+]i elevation or phosphorylation by ERK (23).

The C-terminal tail of SK1 binds proteins, including TRAF2 (tumor necrosis factor receptor-associated factor 2) (24) and PP2A (25). We have suggested that protein binding to the C-terminal tail might have an important role in regulating translocation of SK1 to the PM (11). Indeed, truncation immediately preceding Gly364 in hSK1 to remove the C-terminal tail (21 residues) has been shown to promote constitutive activation and binding to PS-containing lipid raft micro-domains (26) in a manner independent of ERK phosphorylation.

The existence of both phosphorylation-dependent and -independent mechanisms for translocation of SK1 from the cytoplasm to the PM raises a question as to whether there is any mechanistic intersection between the differing modes of translocation at the level of protein structure. This might occur, for example, if the ERK phosphorylation destabilizes an auto-inhibitory C-terminal folding so as to correctly present regions required for binding acidic phospholipids. This would represent a unified mechanism for membrane localisation, promoted either by ERK phosphorylation or by alteration of the position of the C-terminal tail. We have therefore, investigated the structural-functional
properties of SK1 that facilitate its translocation from the cytoplasm to the PM of MCF-7L breast cancer cells using mSK1. This includes defining whether the monomeric or dimeric forms of SK1 localise to different micro-domains in the PM, potentially to facilitate distinct aspects of the enzyme’s pleiotropic signalling functions, and defining the role of the C-terminal tail of SK1 in regulating translocation. Such studies inform on evolutionary conserved structural-functional properties of SK1 and enable identification of possible de-regulated mechanisms controlling translocation of SK1 in pre-clinical murine disease models.

Results

Translocation of SK1 to different micro-domains in the plasma membrane

Confirmation of the expression of WT GFP-mSK1 mutants was by western blot analysis using anti-GFP antibody (Fig. 1A). The treatment of MCF-7L cells with S1P (5 µM, 10 min), PMA (1 µM, 10 min) or carbachol (100 µM, 10 min) induced translocation of endogenous (detected with anti-SK1 antibody) or WT GFP-mSK1 from the cytoplasm to the PM (Fig. 1A). Carbachol binds to M3 and Gq coupled M3 muscarinic receptors in MCF-7 cells (27); S1P has been shown to bind to Gq coupled S1P3 in MCF-7 cells (28), and S1P, PMA or carbachol activate PLD to form PA (29-31). Translocation of SK1 was quantified by measuring membrane immunoreactive or GFP intensity at the PM as an area under the curve (AUC) as described in the Methods. Both endogenous SK1 and WT GFP-mSK1 exhibited significant increases in AUC at the PM after treatment with ligands (Fig 1B, C).

Results were also quantified as the % cells containing PM associated SK1 as described in the Methods. In WT GFP-mSK1 transiently overexpressing MCF-7L cells there appeared to be very little SK1 associated with the PM, with the majority of SK1 localised in the cytoplasm and perinuclear region. Treatment of cells with S1P, PMA or carbachol stimulated translocation of WT GFP-mSK1, such that there was an increase in the % of cells containing PM SK1 (Fig. 1B, C). These responses were observed as a redistribution of SK1 from the cytoplasm/perinuclear regions to the PM. GFP transfected cells stimulated with PMA, carbachol or S1P did not show a redistribution of GFP (Suppl Fig. 1). In order to confirm the identity of the endogenous hSK1, MCF-7L cells were treated with SK1 siRNA, which reduced the intensity of the immunostaining of a 42 kDa protein detected on western blot of cell lysates and decreased immunofluorescence at the PM in response to ligand stimulation of the cells (Fig. 1D).

Dependency on ERK phosphorylation

We next investigated whether the translocation of SK1 to the PM might be via an ERK-catalysed phosphorylation-dependent or -independent mechanism in MCF-7L breast cancer cells. In this case, the pre-treatment of MCF-7L cells with the MEK-1 inhibitor, PD98059 (50 µM, 1 h) decreased activation of ERK-1/2 to all three ligands (Fig. 1A) and reduced the translocation of both endogenous SK1 and WT GFP-mSK1 in response to PMA, but not carbachol or S1P (Fig. 1A-C). This difference in sensitivity to ERK might be due to the finding that PMA is a stronger activator of ERK compared with carbachol or S1P. These findings are supported by studies showing that mSK1 can be phosphorylated by ERK (8). Therefore, carbachol and S1P are ligands that appear to induce the translocation of SK1 to the PM via an ERK-independent mechanism in MCF-7L cells.

Co-localisation studies

The treatment of cells with PMA or carbachol resulted in the translocated SK1 being evenly distributed (spread) in the PM in sub-structures reminiscent of lamellipodia (Fig. 2A). In contrast, S1P promoted translocation of SK1 to focal regions in the PM (Fig. 2A). In order to better define the PM sub-structures in which WT GFP-mSK1 is translocated, co-localisation studies were performed using defined markers of lamellipodia (cortactin), filopodia (fascin) and focal adhesions (paxillin). Pearson correlation coefficients indicated that carbachol and PMA stimulated the co-localisation of WT GFP-mSK1 with cortactin (Fig. 2B) indicating a lamellipodia localisation, while S1P promoted the co-localisation of WT GFP-mSK1 with fascin (Fig. 2B), indicating a filopodia localisation. There was no co-localisation of GFP-mSK1 with paxillin (Fig. 2B), indicating that the sub-structure localisation does not include focal adhesions.

Dependency on PLD 1/2 and Gq

To establish whether the translocation of WT GFP-mSK1 in MCF-7L cells is dependent on formation
of acidic phospholipids, MCF-7L cells were pre-treated with the PLD1/2 inhibitor, FPII. PLD catalyses the hydrolysis of phosphatidylcholine to produce acidic PA and choline. Treatment of MCF-7L cells with FPII (100 nM, 1 h) reduced the translocation of WT GFP-mSK1 in response to carbachol, PMA or S1P (Fig 3A, B). This likely reflects a role for PKC activation, directly (in the case of PMA) or indirectly (as a consequence of Gq/PLC signaling), in the stimulation of PLD and generation of PA. To confirm the involvement of Gq in the stimulated cells loaded with the fluorescence Ca2+ indicator, Cal-520/AM, YM254890 (10 µM) was effective at abolishing Ca2+ mobilization (Suppl Fig. 2).

To provide additional evidence to confirm the role of PLD in regulating translocation of SK1 to the PM, we used CHO cells over-expressing doxycycline-inducible WT PLD2 or K758R inactive PLD2. Western blot analysis confirmed the WT and K758R PLD2 over-expression with doxycycline. Induction of WT PLD2 promoted the translocation of the WT GFP-mSK1. This was not the case with the K758R inactive PLD2 mutant (Fig. 4A, B). These results confirm that PLD2 activity is required for translocation of SK1 to the PM.

Characterization of the monomer-dimer equilibrium
To provide support for the monomer-dimer equilibrium model regulating translocation of SK1 to the PM in a ligand-specific manner, we used proximity ligation assays (PLA) in which WT GFP-mSK1 and WT Myc-tagged mSK1 were over-expressed in MCF-7L cells. This is a ‘dimer formation’ assay and prevention of dimer formation therefore equates with dissociation (accompanied by a reduced signal) under conditions where the dimer pre-exists. In unstimulated cells that were co-transfected with WT GFP-mSK1 and WT Myc-tagged mSK1, there was a significant PLA signal in the cytoplasm indicating that a substantial pool of SK1 is dimeric in the cytoplasm of these cells (Fig. 5A-C). Treatment of cells with either carbachol or PMA virtually abolished the PLA signal, thereby demonstrating that these ligands prevent formation of dimeric SK1 from monomers (Fig. 5B and C). These findings indicate that PMA or carbachol shift the monomer-dimer equilibrium in favor of the monomer. In contrast, a PLA signal was evident in cells treated with S1P, indicating that the dimer is still formed in cells treated with S1P, albeit less than in untreated cells (Fig. 5B and C). Therefore, the monomer/dimer equilibrium can be influenced in a ligand-specific manner. These findings, together with data in Fig. 2, indicate that the monomer translocates to lamellipodia in response to carbachol or PMA, while the dimer might translocate to the filopodia in response to S1P.

Characterization of the translocation of SK1 dimerisation interface mutants
Two site-directed mutagenesis approaches were used to provide additional evidence that SK1 is subject to a monomer/dimer equilibrium and that this can determine the translocation of monomeric or dimeric SK1 to different micro-domains of the PM. As there are no mSK1 crystal structures available at present, our strategy here was guided by analysis of the hSK1 crystal structures, of which there are five currently available (11). These all exhibit a common dimeric assembly of protein molecules, despite the adoption of different packing arrangements for the dimers within the crystal lattices. Dimerisation involves antiparallel partial annealing of an exposed β-strand from the N-terminal domain (NTD) of each protomer about a C2 symmetry axis (Fig. 6A); a similar NTD-NTD dimer organisation is seen almost universally across currently available crystal structures for related DAGK cat family proteins (11). The extent of interfacial surface contact is comparatively modest however (ca. 780 Å2 buried solvent accessible surface area per protomer), which might be consistent with the formation of a mobile (and potentially dynamically regulated) equilibrium between monomeric and dimeric states of SK1 in a cellular context. Analysis of the dimerisation interface for hSK1 suggests contributions from both
hydrophobic surface engagement and the establishment of a highly organized polar network. There are subtle differences in the amino acid sequence for mSK1, but homology models (Fig. 6B) suggested that a lysine (K49) is likely essential for establishment of the polar interaction network in the murine enzyme through cross-dimer salt bridging. Thus, K49E charge reversal mutation is predicted to cause severe charge opposition across the dimer interface and, therefore, to impair the dimerisation capacity of the enzyme. In order to achieve the converse effect and stabilise the dimeric state, we also sought to generate a disulfide bridge. For that purpose, we introduced an I51C mutation in the strand sequence (50-LIL-52, corresponding to 51-LML-53 in hSK1) that forms the hydrophobic core of the dimerisation interface. Our models suggested that the Cα-centres of I51 in mSK1 likely experience contact in the dimer and that this might therefore be a reasonable site for an engineered disulfide bridge (Fig. 6C). Although cross-strand disulfides in certain contexts can be strained (33 and references therein), we reasoned that a disulfide might be favourably deployed here without distorting the protein structure because the two symmetry related strands diverge from one another on either side of I51. Therefore, it appears reasonable that a C51:C51 disulfide bridge across the dimer symmetry axis would not attract significant penalties associated with distortion to side-by-side strand packing.

Confirmation of the expression of K49E and I51C GFP-mSK1 mutants was by western blot analysis using anti-GFP antibody (Fig. 7A). The treatment of MCF-7L cells with carbachol or PMA promoted the translocation of the K49E mutant to lamellipodia, albeit this was reduced compared with WT SK1 (Fig. 7A-C). Moreover, the treatment of MCF-7L cells with S1P also promoted the translocation of the K49E mutant to lamellipodia in the PM. This contrasts with the WT enzyme, which translocates to filopodia in response to S1P. The treatment of MCF-7L cells with S1P, PMA or carbachol induced the translocation of the I51C mutant to filopodia, with no localisation to lamellipodia (Fig. 7A-C). Together with the redirection of the K49E mutant to lamellipodia in response to S1P, these findings suggest that S1P and carbachol do not influence translocation of SK1 by regulating the formation of lamellipodia and/or filopodia per se. Rather, the findings confirm that the mutants exhibit different micro-domain localisation in the PM and this is governed in a ligand-specific manner.

We used the PLA to establish the dimerisation status of the mutants. In this case, the WT GFP-mSK1 in the pairing with WT Myc-tagged mSK1 was replaced by the GFP mutants in order to establish whether a PLA signal can be generated. The expression levels of GFP-mSK1 were similar in each combination and this was also the case for Myc-tagged mSK1 (Fig 8A). The pairing of K49E GFP-mSK1 mutant with WT Myc-tagged mSK1 produced a low PLA signal and this was considerably less than the PLA signal generated by the WT GFP-mSK1 and WT Myc-tagged mSK1 pairing (Fig. 8B and C). These findings suggest that the loss of one of the two symmetry-related salt bridges involving K49 at the modelled mSK1 dimerisation interface (Fig. 6B) is sufficient to substantially weaken dimerisation but not to completely ablate formation of a WT Myc-tagged mSK1/K49E GFP-mSK1 heterodimer. The loss of both salt bridges and introduction of greater charge opposition is likely to weaken affinity of the protomers for each other yet further, however, such that homodimer formation with the K49E GFP-mSK1 mutant would be even more severely compromised, resulting in a species that is essentially monomeric when expressed in MCF-7L cells and which we show here to be localised exclusively to lamellipodia in response to carbachol or PMA challenge (Fig. 7A-C).

Over-expression of WT Myc-tagged mSK1 with I51C GFP-mSK1 mutant produced a low PLA signal (Fig. 8B and C). The reduced PLA signal is consistent with the I51C mutant being already locked in as a dimer and therefore unable to form a dimer with WT SK1. This is supported by the finding that the I51C mutant does not behave like a monomer as exemplified by the K49E mutant which localizes to lamellipodia, while the I51C mutant is localized in filopodia (Fig. 7A-C). These findings are also in line with S1P maintaining the WT dimer and promoting its localization to filopodia, while PMA/carbachol promote formation of WT monomer and induce its localization to lamellipodia. Further studies concerning the I51C mutant are required.
A small amount of WT Myc-mSK1/I51C GFP-mSK1 dimer is formed, but this is considerably reduced compared with WT GFP-mSK1/WT Myc-mSK1 dimers and may reflect redox conditions controlling the integrity of disulfide bonds in the putative I51C GFP-mSK1/I51C GFP-mSK1 homodimer.

**Characterization of translocation properties for C-terminally truncated mutants (T1-T5)**

To investigate whether the C-terminal tail of SK1 is likely to have an important role in regulating its translocation to the PM, truncated forms of GFP-mSK1 were constructed. In these truncates (T1-T5, Fig. 9A) blocks of five amino acids were progressively removed from the C-terminus, with the exception of T4, which had nineteen amino-acids removed in total. Truncates T1-T5 were separately over-expressed in MCF-7L cells, which were then stimulated with carbachol or S1P or PMA. In earlier studies, C-terminal cleavage before G364 in hSK1 was shown to promote binding to acidic PS-containing lipid rafts and cause constitutive activation (26). The addition of just four residues, 364-GXXX-367, restored the reduced basal activity level of hSK1. In mSK1 G364 is conserved (as G362), but the next three residues differ in identity from hSK1. However, Hengst and co-workers (26) had also shown that the restoration of the lower basal enzyme activity with the four-residue extension (364-GXXX-367) was independent of residue identity, suggesting that it is the peptide backbone in this region rather than specific side chains that may be important. The effect of the added sequence may potentially be due to capping at the C-terminal end of helix-5 by the 364-GXXX-367 peptide backbone (11), but this has not been firmly established to date because G364 also represents the C-terminal limit of crystallographic structural definition in hSK1 at present. The G362 residue was retained in our exploratory T4 truncate but removed in the T5 construct. Confirmation of the expression of WT and T1-T5 GFP-mSK1 mutants was by western blot analysis using anti-GFP antibody (Fig. 9B). The translocation of the T1 mutant, lacking the last 5 amino acids at the C-terminus (PPEEP) that are conserved in murine and human SK1, was severely reduced in response to carbachol or S1P, while translocation in response to PMA was unaffected (Fig. 9C, D).

The T1 mutant failed to translocate to the PM within the 10 min stimulation period with carbachol, indicating that it does not associate and ‘fall off’ the PM at a faster rate compared with the WT SK1 during this time frame, but simply exhibits reduced translocation to the PM (Fig. 10). In contrast, the T2-T5 mutants lacking 10-25 amino acids all translocated to the PM in response carbachol, S1P or PMA and these translocations were inhibited by FPI1 or by YM254890 (Suppl Fig. 3A, B, 4A, B, 5A, B and Fig. 6A, B). These findings indicate the both PA and G1 are still required for the translocation of T2-T5 mutants, in common with the WT enzyme.

**S1P measurements**

To confirm the functional competence of the SK1 mutants in terms of their ability to produce S1P and to exclude an effect of mutation on activity, we measured S1P levels in MCF-7L cells over-expressing WT, K49E, I51C and T1-T5 mutants using MS analysis. All the mutants and WT enzyme produced the same significant or approaching significant increase in S1P compared with GFP-transfected cells, with the exception of the T5 mutant, which failed to increase S1P levels above GFP-transfected cells (Fig. 11). This finding likely reflects the fact that 357-LYMVCG-362 sequence, which is present in T1-T4 but deleted in T5 (Fig. 9A), contributes to a critical inter-domain twisted strand pair that is essential for the correct functional interaction of ATP-binding NTD and sphingosine-binding CTD halves of the enzyme (vide infra, Discussion). The helix-5 and T-loop residues are the same between mSK1 and hSK1, consistent with evolutionary conservation of this key structural element in SK1. Similar significant increases in dihydros1P levels were observed for WT and mutants, with the exception of T5 (Fig. 11).

**Discussion**

We have shown here that monomeric or dimeric WT SK1 localizes to specific lipid micro-domains in the PM dependent on the GPCR signaling cue (e.g. carbachol, S1P). There are at least two different types of PM micro-domain, notably lamellipodia and filopodia. Here we observed that carbachol stimulated Gα-coupled AchR signaling in MCF-7L cancer cells promotes translocation of monomeric WT SK1 to lamellipodia. In contrast, Gα-coupled S1P/S1P3 signaling induces translocation of dimeric
WT SK1 from the cytoplasm to filopodia. These data are based upon the findings that only monomeric SK1 (no dimeric SK1 is detected) is present in cells treated with carbachol or PMA, while dimeric SK1 is present in MCF-7L cells treated with S1P (Fig. 5), and that these forms translocate to lamellipodia or filopodia respectively (Fig. 2). This model is supported by results using SK1 constructs engineered with mutations intended to destabilise (K49E) and stabilise (I51C) the dimerisation interface. In this case, the K49E monomer localises to lamellipodia in response to PMA and carbachol, while the I51C mutant moves to filopodia in response to S1P (Fig. 7).

In MCF-7L cells treated with S1P, both monomeric and dimeric WT SK1 are present, and this raises the question as to why the monomeric SK1 does not translocate to lamellipodia in addition to filopodia. This might suggest that the translocation of monomeric WT SK1 to lamellipodia is threshold dependent and that S1P does not breach this threshold, while carbachol and PMA do so, as supported by the PLA data obtained in this study.

Therefore, we present here a new concept that is important because one might predict that the dimeric SK1 will exhibit more avidity for the PM than the monomeric form (based on the topographically coordinated presentation of a larger number of positively charged centres and the presence of two surface-exposed hydrophobic patches for LBL-1 loops of the dimer). This might conceivably impact on the residence time that the monomer or dimer have at the PM and therefore on the magnitude of the S1P signal generated, which in turn could influence the potentially different biological effect produced in both a spatial and temporal context.

Membrane localisation of SK1 is known to be sensitive to enrichment in acidic phospholipids, such as PA produced by PLD (17), and influenced by physical aspects, such as membrane curvature (12). This leaves the question of how the monomeric and dimeric forms of SK1 can sense different micro-domains in the PM. This might occur because higher positive charged density in the dimer versus monomer might enable the dimer to bind with more avidity to lipid micro-domains in which acidic phospholipid content is limiting for the monomer. Alternatively the differential localisation might be governed by the possibility that the dimeric assembly could be more conducive to curvature-sensitive binding to the membrane in filopodia, while the monomer might bind in a curvature-insensitive manner in the lamellipodia. These possibilities require further investigation.

The investigation into the role of the C-terminal tail of SK1 in regulating translocation revealed that the T1 mutant, that lacks the last 5 amino acids at the C-terminus, is defective for translocation while the T2 mutant, that lacks the last 10 amino acids, is able to translocate to the PM. From these findings it appears that amino acids 6-10 preceding the C-terminus, which are present in T1 but not T2, may hold the enzyme in a ‘locked’ state that prevents translocation and either requires the C-terminal PPEEP sequence to be present for release under conditions of Gq drive or requires conditions that drive ERK activation for the lock to be overturned; in the latter case the PPEEP terminus appears non-critical (e.g. PMA/T1). Analysis of the available crystal structures for SK1 suggests that the position for attachment of the C-terminal tail together with the location of the R-loop, which contains the phosphorylation site for ERK, are well placed to jointly orchestrate inter-domain movement that could regulate the alignment of membrane engagement determinants in the NTD and CTD.

To date it has not been possible to obtain SK1 crystals with the intact C-terminal tail, and the available structures are therefore truncated after G364 in hSK1 (corresponding to G362 in mSK1). The disposition of the tail residues remains to be defined, therefore. It is clear, however, that in the crystallographically observed conformation the C-terminal tail threads through a narrow cleft between the NTD and a long twisted β-strand pair that connects the NTD to the CTD (Fig. 12, green ribbon). The ability to be able to adopt this threaded state suggests a folding mechanism that may involve initial strand alignment in a less twisted state followed by inter-domain rotation. Thus, we cannot at present rule out the possibility that translocation between the cytosol and PM may involve significant inter-domain rotational movements about the twisted pair. A potential explanation for the influence of the C-terminal tail on translocation in our study, then, may be that the putative locking sequence (amino acids 6-10 preceding the C-
terminus) folds across the CTD to actively maintain a conformational arrangement for the two domains that keeps the membrane interfac ing determinants out of alignment for coordinated engagement of the membrane. Binding of a protein partner to the terminal acidic polyproline sequence (PPEEP) under conditions of Gq drive may displace the C-terminal tail from its locking position to facilitate the particular inter-domain arrangement with coordinated NTD and CTD membrane binding determinants. In principle, phosphorylation of S225 under conditions of PMA drive might elicit a structural transition in the regulatory R-loop (Fig. 12, cyan ribbon) that displaces the C-terminal tail without the need for sequestration of the PPEEP C-terminus. Controlled phasing of membrane interfac ing determinants in the NTD and CTD by these mechanisms might operate in both the monomer and the dimer to regulate translocation to the PM but with the dimer providing an extended interface that may influence affinity and sensitivity to membrane curvature. Further work is required to establish a detailed structural basis for the role of the C-terminal tail in regulating membrane localisation.

We conclude that the C-terminal tail of SK1 can regulate the translocation of SK1 to different microdomains in the PM in a ligand specific manner that is affected by the monomer/dimer status of the enzyme. The precise mechanistic basis for perturbation of the monomer/dimer equilibrium requires further investigation. However, we propose that this differential localisation of monomer versus dimer likely increases the repertoire of function and endows the SIP that is formed, with spatial and temporal pleiotropic signaling properties. This may provide new options for treating disease by blocking the C-terminal tail function of SK1 to eliminate translocation and activation of the enzyme. In addition, the existence of a monomer/dimer equilibrium might be used to inform on therapeutic targeting strategies in cases where, for example, the dimeric SK1 might drive disease pathology, while the monomeric form does not or vice versa.

**Experimental procedures**

**Materials**

Cell culture reagents including high glucose Dulbecco’s modified Eagle’s medium (DMEM), antibiotics and LipofectAMINE®2000 were purchased from Invitrogen (Paisley, UK). S1P was from Avanti Polar Lipids (Alabaster, AL), YM254890 from Caltag Medsystems (Buckingham, UK). FIPI (N-[2-[4-(2,3-dihydro-2-oxo-1Hbenzimidazol-1-yl)-1-piperidinyl]ethyl]-5-fluoro-1Hindole-2-carboxamide hydrochloride) from Cayman Chemical Company (Ann Arbor, MI) and PD98059 from Sigma-Aldrich Company Ltd (Gillingham, UK). Antibodies employed were anti-GAPDH (sc-47724), anti-GFP (sc-9996) and anti-phosphoERK1/2 (sc-7383) from Santa Cruz (through Insight Biotechnology Ltd (Wembley, UK)), anti-ERK2 (#610104) from BD Biosciences (Wokingham, UK), anti-HA tag antibody (H3663) and anti-actin (A2066) were from Sigma-Aldrich Company Ltd. Anti-fascin (#54545), anti-paxillin (#92542) and anti-cortactin (#3503) antibodies were purchased from Cell Signaling Technology (London, UK). The anti-SK1 antibody was custom synthesised by Abgent using the antigens H2N-CPRGKGA3LFRSH-CONH2 and H2N-CPRGKGA3LFRSH-CONH2 (34). Vectorshield with DAPI was from Vector Laboratories UK (Peterborough, UK). Plasmid DNA used in this study was purified using endotoxin free plasmid preparation kits and their sequences validated by Sanger sequencing (GATC, Cologne, Germany). Primers were synthesised by Integrated DNA Technologies, BVBA (Petersbrough, Belgium).

**Cell Culture**

MCF-7L cells were maintained in a humidified incubator at 37 °C, with CO2 (5% (v/v)) in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with fetal calf serum (FCS) (10% (v/v)), penicillin (100 U/mL) and streptomycin (100 µg/mL) (complete DMEM media). PLD2-inducible Chinese-hamster ovary (CHO) cells were maintained in Ham’s F12 medium respectively, supplemented with 10% (v/v) FCS (and penicillin (100 U/mL)/streptomycin (100 µg/mL)). In all cases, cells were deprived of serum for 24 h prior to experimentation.

**Molecular biology**

Truncations and site-directed mutagenesis of a construct encoding GFP-tagged wild type mouse SK1 (WT GFP-wSK1) was by PCR using pFulUltra II fusion Hotstart high fidelity DNA polymerase (Agilent Technologies LDA UK Limited (Stockport, UK)) (95 °C for 2 min to activate the polymerase,
followed by thermal cycling of 95 °C for 20 seconds, 60 °C for 20 seconds, 72 °C for 105 seconds, 20 cycles) and specific primers. Truncations were of 5, 10, 15, 19 or 25 C-terminal amino acids (termed GFP-mSK1-T1 to T5, respectively).

Primer Sequences (5’-3’)
- GFP-mSK1-T1, Forward: ACTCCGGCGGGGCGATAACGAAGAAT TACT, Reverse: GATGTCGCTTTCTCTCTTG
- GFP-mSK1-T2, Forward: CACCCGCTTCTGGCTTGTGGG
- GFP-mSK1-T3, Forward: AGTGATACCCGATCCCTGAG
- GFP-mSK1-T4, Forward: CGGAGCTCAGCAGAAG
- GFP-mSK1-T5, Forward: CCGCCTCTCTCTTG
- GFP-mSK1-K49E, Forward: CAGGATCAACCTTGAAGCTTCACT
- GFP-mSK1-I51C, Forward: GAGATACCTTAACTTTG

Transfections
MCF-7L cells, plated for 24 hours or until 70% confluent (and on coverslips, where indicated), were transfected with of DNA (1 µg), as indicated in legends, using LipofectAMINE® 2000 according to the manufacturer’s instructions. The transfection mixture was added drop-wise to cells and incubated for 24 hours. Cells were further incubated in serum free DMEM media for 24 hours prior to treatment (see figure legends).

Treatments
Cells were treated with either vehicle, carbachol (100 µM final), S1P (5 µM), PMA (1 µM) for 10 min. Where indicated (see figure legends), cell were pre-incubated with PD98059 (50 µM) (inhibitor of MEK1 and MAP kinase cascade) for 1 hour, YM254890 (Gq inhibitor) (10 µM) for 30 min or FIP (PLD-1/2 inhibitor) (100 nM) for 1 hour.

Western blotting
Cells were harvested by removal of the media and addition of Laemmli sample buffer (200 µL; sodium dodecyl sulphate (SDS) (0.5% (w/v)), Tris HCl (125 mM, pH 6.7), ethylenediaminetetraacetic acid (1.25 mM), sodium pyrophosphate (0.5 mM), dithiothreitol (50 mM), bromophenol blue (0.06% (w/v)) and glycerol (12.5% (v/v)) before homogenisation using a 23-gauge hypodermic needle and syringe. Samples were analysed by SDS-PAGE in parallel with pre-stained molecular weight markers and western blotted for proteins of interest. Similar protein loading between samples was determined by re-probing for either ERK2 or GAPDH.

Immunofluorescence microscopy
Cells grown on 13 mm autoclaved glass cover slips were transfected, if required, as above and incubated in serum free DMEM media for 24 hours prior to treatment (see figure legends). Cells were fixed with formaldehyde (3.7% (v/v) in phosphate buffered saline (PBS)) for 15 min and permeabilised using Triton X-100 (0.1% (v/v) in PBS) for 2 min. For the detection of transfected forms of GFP-mSK1, the coverslips were washed briefly with PBS and mounted on glass slides using VECTASHIELD hard-set anti-fade mounting medium with DAPI. For the detection of endogenous proteins, the coverslips were incubated with blocking buffer (FCS 5% (v/v) and BSA 1% (w/v) in PBS) for 30 min before
incubation with primary antibody (1:50) in blocking buffer overnight at 4 °C. After briefly washing in PBS, coverslips were incubated with FITC- or TRITC-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibody (1:50) in blocking solution for 1 hour at room temperature. Coverslips were washed in PBS before mounting on glass slides using VECTASHIELD with DAPI. Samples were imaged for DAPI and GFP (FITC settings) using an epifluorescent upright microscope (Nikon, Eclipse E600) with 40X oil lens accompanied with WinFluor imaging software.

Quantification of GFP-mSK1 translocation
For quantification of % of cells displaying lamellipodia or filopodia/foci localisation of GFP-mSK1, immunofluorescence images of 15 cells (randomly chosen) were acquired from three or more experiments. These were assessed (non-blinded) for evidence of GFP-mSK1 translocation to the PM and whether this was predominantly lamellipodia or localised to filopodia/foci. ImageJ (Scion Corporation, Frederick, MD) analysis of raw images acquired for GFP alone (no DAPI signal) was also used to quantify PM translocated GFP-mSK1. The segmented line tool was used to measure the pixel intensity (on a scale of 0-256 (black-white), corresponding to increasing amounts of GFP-mSK1) for a distance of 500 pixels at the PM. Data from 5 cells were combined in series (distance of 2500 pixels). The average pixel intensity (2500 pixels) of 5 control cells was subtracted as background fluorescence for each experiment. All images were of similar brightness and contrast. The area under the curve (AUC) of a point-to-point plot was calculated as a quantitative measure of GFP-mSK1 translocation for a given treatment or SK1 mutant.

Proximity ligation assay
Duolink® In situ Orange proximity ligation assays (DUO92102, Sigma-Aldrich) were carried out in MCF-7L cells transfected with WT GFP-mSK1 or GFP-mK48E or GFP-mI51C SK1 mutant constructs and/or Myc-tagged mSK1. MCF-7L cells were seeded in 12-well plates containing 13 mm diameter glass coverslips prior to transfection as previously detailed. Interaction was first determined in unstimulated co-transfected cells and upon treatment with carbachol (100 µM), S1P (5 µM) and PMA (1 µM) for 10 min. Further experiments were carried out to assess interaction between Myc-mSK1 (WT) and GFP-SK1 mutants (K49E and I51C). Cell fixation, permeabilization and blocking was carried as detailed in the Immunofluorescence Microscopy section. Coverslips were incubated with rabbit anti-GFP (Clontech, Takara) and mouse anti-Myc-Tag primary antibodies overnight (1:500). PLA were performed as per manufacturer's instructions using the Duolink In Situ (orange) range of detection reagents, wash buffers and PLA probe kits. All incubation steps were carried out in a humidity chamber 37 °C. After primary antibody incubation, cells were washed twice in Duolink wash buffer A for 5 min then incubated in rabbit PLUS and mouse MINUS Duolink PLA probes used at a 1:5 dilution in Duolink Antibody Diluent at 37 °C for 1 hour. Following incubation, cells were washed twice in Duolink wash buffer A for 5 min then incubated in Duolink ligation buffer (1:5) containing 1 U ligase at 37°C for 30 min. Coverslips were washed twice in buffer A for 5 min and incubated in Duolink amplification buffer (1:5) containing 0.5 U polymerase at 37 °C for 100 min (photosensitive reaction). Cells were washed in Duolink wash buffer B for 10 min at room temperature followed with a final wash in 0.01X buffer B for 1 min. Cells were counterstained with DAPI (500nM) for 5 min, washed three times with PBS, and the coverslips were mounted on to glass microscope slides with Mowiol. PLA positive cells were visualised using a Leica TCS SP8 confocal microscope at x63 magnification with an oil-immersion lens (PLA Orange wavelengths ex 554 nm; em 576 nm). GFP transfected cells were used as a hallmark of successful transfection (FITC settings). PLA experiments were carried out blinded (RDRB transfected cells and coded experimental sample sets). Image acquisition was carried out using Leica LAS software, with images processed via ImageJ (Scion Corporation, Frederick, MD). Cellular PLA signal intensities were measured for each positive cell, with cell populations averaged for each treatment group (50 cells/treatment) and presented as mean ± SEM with analysis carried out using Graphpad Prism 9. Indirect immunofluorescence was carried out in parallel to PLA to demonstrate successful co-transfection and expression of target proteins of interest as described previously.

Lipidomics
Single-phase lipid extraction and derivatization
A single-phase butanol:methanol (1:1, BuMe) lipid extraction protocol was used to extract S1P from cells. Cell pellets were mixed with 400 µL of BuMe and 200 µL of internal standard (ISTD) solution containing S1P d18:1 ¹³C₂D₂ in BuMe. Samples were mixed with a vortex for 10 seconds and sonicated with ice in an ultrasonic bath for 30 minutes. Samples were then centrifuged at 14,000 g for 10 minutes at room temperature. A volume of 250 µL of supernatant from each sample was transferred to eppendorf tubes, dried completely in a speed-vac and resuspended in 90 µL of BuMe. S1P species were derivatised as described in Narayanaswamy et al. (35). A volume of 30 µL TMS-diazomethane (TMS) was added to the resuspended extracts which were incubated in a thermomixer at 1,000 rpm for 20 minutes at room temperature. To stop the reaction, 1 µL of glacial acetic acid was added to each sample. Samples were mixed with a vortex mixer and centrifuged at 16,000 g at room temperature. The supernatants were transferred to the MS vial for LC-MS analysis.

**Sphingoid base-1-phosphates (SIP) analysis**

A chromatographic separation was performed on a Waters ACQUITY UPLC BEH HILIC, (130 Å, 2.1 x 100 mm, 1.7 µm) column, thermostatted at 60°C in an Agilent 1290 UHPLC system. The flow rate was 400 µL min⁻¹ with mobile phase A composed of acetonitrile-25 mM ammonium formate buffer (50:50, v/v) and mobile phase B composed of acetonitrile-25 mM ammonium formate buffer (95:5, v/v). Mobile phase A and B were mixed to create the following gradient: 99.90% B at 0 min to 40% B at 5.00 min, 10% B at 6.50 min and re-equilibrated at 99.90% B from 6.60 min to 9.60 min. The total run time was 9.60 min. An Agilent 6495 QQQ was used for targeted measurements. The AJ5 ESI source parameters for the MS were: dry gas temperature and flow 200°C and 12 L min⁻¹ respectively, nebulizer pressure 25 psi, sheath gas temperature and flow were set to 400°C and 12 L min⁻¹ respectively, capillary voltage and nozzle voltage set to 3500 V and 500 V respectively, and the delta EMV 200 V. Positive high/low pressure RF was set to 200/110.

**Data Analysis**

The acquired MS data were analyzed using Agilent MassHunter software version B.08.00. The signal to noise ratios (S/N) were calculated using the raw peak areas in the study samples and processed blanks (PBLK). Lipids that had S/N < 10 and a CoV > 30% (calculated from a pooled QC analysed every 5 samples) were discarded. Internal standards were used to normalize the raw peak areas using corresponding quantifier/qualifier transitions. All species were normalized to S1P d18:1 ¹³C₂D₂. The values after normalization to IS were further normalized to the protein amount. The MS was operated in positive ionization mode and a multiple reaction monitoring method (MRM) was set up for the analysis. For each SIP, two product ions at m/z 60.08 (quantifier) and m/z 113 (qualifier) generated from the TMS derivatized tetra-methylated form of S1P were monitored.

**Calcium measurements**

Intracellular Ca²⁺ was monitored using the Ca²⁺ sensitive fluorescent indicator, Cal-520 (36, 37). For this, MCF-7L breast cancer cells were plated in ibidi µ-slide 8 well culture dishes and left to adhere for 24 hours (37°C, 5% CO₂). After 24 hours the media was replaced with serum-free media for 24 hours. Cells in the Ibidi chamber were incubated in DMEM containing Cal-520/AM (5 µM with 0.04% Pluronic F-127 and 0.26% DMSO) for 30 mins at 37°C in the dark. Ca²⁺ imaging was performed using an inverted fluorescence microscope (TE2000U; Nikon, Tokyo, Japan) equipped with a 40X objective (oil immersion, 1.3 NA; Nikon S Fluor). Fluorescence emission was recorded at 10 Hz using a large-format (1024 x 1024 13 µm pixels) EMCCD camera (iXon 888; Andor, Belfast, UK). Cal-520/AM was excited with 488 nm wide-field epifluorescence illumination provided by a monochromator (Photon Technology International/Horiba UK, Ltd, Stanmore, UK). Ca²⁺ activity was recorded at room temperature for 5 minutes. A stable baseline was established for the first 60s of the recording before the addition of carbachol (100 µM). To examine the effects of the selective Gq11 inhibitor YM254890 (10 µM), MCF-7L cells were incubated in YM254890 for 30 mins at 37°C before the addition of carbachol. Temporal Ca²⁺ signals were extracted from the raw fluorescence intensity image stacks, using 30-pixel (~4 µm) diameter circular ROIs manually positioned at the centre of each cell. Ca²⁺ signals were analysed using a custom Python-based analysis suite as described previously (41, 42). The average initial Ca²⁺ peak response evoked by carbachol in the absence and presence of YM254890 was analysed in Graphpad Prism v7.01 (GraphPad, San Diego, CA, USA).
**Statistics**
Data is presented as mean ± SEM and was statistically analysed using GraphPad Prism 7 using one or two-way ANOVA and Tukey's multiple comparison test or Bonferroni’s post hoc test or by unpaired two-tailed t test.

**Pearson correlation coefficients**

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**Data Availability Statement:** All data are contained within the manuscript.

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Figures

Fig. 1 Effect of the MEK-1 inhibitor, PD98059 on the translocation of endogenous SK1 and WT GFP-mSK1 in MCF-7L cells. MCF-7L cells expressing endogenous SK1 and WT GFP-mSK1 were pre-treated with and without PD98059 (50 μM, 1 hour) or SK1 siRNA (200 nM, 24 h) prior to S1P (5 μM) or PMA (1 μM,) or carbachol (100 μM) for 10 min. Cells were processed (see methods) and mounted with DAPI to stain DNA (blue). (A) 40x oil magnification photomicrographs of cells expressing endogenous SK1 or WT GFP-mSK1 (detected with an anti-SK1 antibody and FITC-conjugated secondary antibody or by GFP immunofluorescence, respectively). Inset is a western blot probed with anti-GFP antibody or P-ERK antibodies showing the over-expression of WT GFP-mSK1 and inhibition of ERK-1/2 phosphorylation by
PD98059. Re-probing with total ERK-1/2 or GAPDH is used to confirm similar protein loading. Representative results of 3 independent experiments. (B and C) Membrane intensity measurements were made from 5 individual MCF-7L cells and stitched together (see methods) (n=5) for endogenous SK1 (B) and WT GFP-mSK1 (C). (B) The bar graph represents the AUC of the total level of endogenous SK1 translocation (n=5); ** p < 0.01 PMA alone vs PMA with PD98059; * p < 0.05, ** p < 0.01 and *** p < 0.001 for stimulus vs control for endogenous SK1 (two way ANOVA with Tukey post hoc test). (C) The upper bar graph represents the AUC of transfected WT GFP-mSK1 translocation (n=5). The lower bar graph represents the % of cells containing translocated WT GFP-mSK1 (n=3); ** p < 0.01, **** p < 0.0001 PMA alone vs PMA with PD98059; * p < 0.05, ** p < 0.01 and **** p < 0.0001 for stimulus vs control transfected WT GFP-mSK1 (two way ANOVA with Tukey post hoc test). (D) 40x oil magnification photomicrographs of cells showing the effect of SK1 siRNA on endogenous SK1 expression. The bar graph represents the % of cells (n=3) containing translocated endogenous SK1. +++ p < 0.0001 for control scrambled vs stimulated scrambled, **** p < 0.0001 for control scrambled vs stimulated scrambled (two way ANOVA with Tukey post hoc test). The second bar graph represents the AUC of the total level of endogenous SK1 translocation (n=5). * p < 0.01 and **** p < 0.0001 for control scrambled vs stimulated scrambled, * p < 0.05, *** p < 0.001 and **** p < 0.0001 for SK1 siRNA vs scrambled (two way ANOVA with Tukey post hoc test).

Fig. 2. Co-localisation of SK1 with lamellipodia and filopodia markers. MCF-7L cells over-expressing WT GFP-mSK1 were treated with S1P (5 µM) or PMA (1 µM) or carbachol (100 µM) for 10 min. Cells were processed and mounted with DAPI to stain DNA (blue). (A) Bar graph representing the % of cells containing translocated WT GFP-mSK1 in lamellipodia and filopodia micro-domains in the PM in response to stimulus (n=4); **** p < 0.0001 treated vs control (two way ANOVA with Tukey post hoc test). (B) 40x oil magnification photomicrographs of cells over-expressing WT GFP-mSK1 detected with GFP and cortactin (lamellipodia marker), fascin (filopodia marker) and paxillin (adhesion foci marker) detected with respective specific antibodies. Representative results of 3 independent experiments. Also shown is a bar graph of the Pearson Correlation Coefficients of co-localisation. * p < 0.01, *** p < 0.001 and **** p < 0.0001 for Pearson Correlation Coefficients for stimulated vs control (increase/decrease) using two way ANOVA with Tukey post hoc test.

Fig. 3 Effect of the PLD inhibitor, FIPI or the Gq inhibitor YM254890 on the translocation of WT GFP-mSK1 in MCF-7L cells. MCF-7L cells over-expressing WT GFP-mSK1 were pre-treated with and without FIPI (100 nM, 1 h) or YM254890 (10 µM, 30 min) prior to S1P (5 µM) or PMA (1 µM) or carbachol (100 µM) for 10 min. Cells were processed and mounted with DAPI to stain DNA (blue). (A) 40x oil magnification photomicrographs of cells over-expressing WT GFP-mSK1 detected with GFP. Representative
results of 3 independent experiments. (B) The bar graphs (right) represent the AUC of transfected WT GFP-mSK1 translocation (n=5) and (left) the % of cells containing translocated WT GFP-mSK1 (n=3); * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 for stimulus alone vs stimulus with either FIPI or YM254890; + p<0.05, ++ p<0.01, +++ p<0.001 and ++++ p<0.0001 for stimulus vs control transfected WT GFP-mSK1 (two way ANOVA with Tukey post hoc test).

Fig. 4. PLD2 dependency of SK1 translocation. WT HA-PLD2-inducible or K758R inactive HA-PLD2-inducible CHO cells were transiently transfected with WT GFP-mSK1 construct and then treated with 2 µg/ml doxycycline for 24 h. (A) 40x oil magnification photomicrographs of cells over-expressing WT GFP-mSK1 detected with GFP. Representative results of 3 independent experiments. Also shown is a western blot of cell lysates with anti-HA antibody, detecting induced PLD2, and ERK-2 (loading control). (B) The bar graphs represent the AUC of transfected WT GFP-mSK1 translocation (n=5) and the % of cells containing translocated WT GFP-mSK1 (n=3). +++ p<0.001 doxycycline vs control and *** p<0.001 for WT PLD2 vs K758R PLD2 in doxycycline-treated cells (two way ANOVA with Tukey post hoc test).

Fig. 5 Conditional changes in protein interaction detection between WT Myc-mSK1 and WT GFP-mSK1 using Duolink Proximity Ligation Assay (PLA). (A) Over-expression of WT Myc-mSK1 or WT GFP-mSK1 was confirmed in MCF-7L cells by fluorescence microscopy prior to Duolink PLA to assess protein interaction. PLA was carried out to assess differences in protein interaction between WT Myc-mSK1 and WT GFP-mSK1 (unstimulated control) and upon treatment with either S1P (5 µM) or PMA (1 µM) or carbachol (100 µM) for 10 min. Untransfected cells (No-TF) and cells expressing only WT GFP-mSK1 or WT Myc-mSK1 were used as negative controls. Cells were processed for PLA and mounted with DAPI to stain DNA (blue). (B) Quantitative analysis of the cellular PLA signals was carried out. The bar graphs represent the PLA signal mean gray value (mean ± SEM) with experiments carried out in triplicate (30 cells total per treatment group shown, n=3) ** p<0.01 and *** p<0.001 for stimulated vs control in WT Myc-mSK1/WT GFP-mSK1 transfected cells (one way ANOVA with Bonferroni post hoc test). (C) Representative PLA images are shown for each treatment group. Scale bar = 20 µm. Results are representative of 3 independent experiments.

Fig. 6 SK1 dimerisation interface and rationale for site-directed mutagenesis. (A) Crystal structures of human SK1 exhibit head-to-head dimerisation with partial annealing of an exposed strand edge (β2) from each protomer about a C2-symmetry axis. A key salt bridge network comprising E67 and R71 in hSK1 contributes to the dimerisation interface, as illustrated here from a 1.8 Å resolution structure (PDB: 4V24) (21) with the dimerisation symmetry axis marked by an asterisk. The positions of bound Sph (yellow surface)
and Mg-ADP (green sphere/cyan surface) are shown superimposed from separate crystal structures 4VZB/4VZD (19). Packing of alternating hydrophobic residues (51-MLM-53 in hSK1) along the annealed β2-strand edges also contributes significantly to the dimerisation interface (M52 shown here in stick). (B) In mSK1 the glutamic acid of the dimerisation salt bridge network is conserved (as E66), but the arginine is replaced by histidine (H70). The additional replacement of a threonine (T50) in hSK1 by a lysine (K49) in mSK1, however, suggests the existence a surrogate E66-K49:E66′-K49′ salt bridge network to maintain the dimerisation interface in mSK1, as illustrated here (mSK1 homology model). Coincident replacement of M52 in hSK1 with a shorter, branched side chain residue in mSK1 (I51, marked) creates space for the lysines and is also consistent with adoption of the postulated surrogate salt bridge network. Based on this model we surmised that K49E charge reversal mutation would disrupt the salt bridge network (black dotted lines) to destabilise the dimerisation interface. (C) The mSK1 homology model of panel (B) indicated that the Cγ-centres for the I51 and I51′ residues of the dimer likely lie within van der Waals contact of one another, thereby suggesting a reciprocal strategy to stabilise the dimerisation interface by means of an engineered disulfide bridge (as modelled here) through the introduction of I51C substitution.

Fig. 7 Translocation of WT, K49E and I51C GFP-mSK1 in MCF-7L cells. MCF-7L cells over-expressing WT GFP-mSK1 or K49E GFP-mSK1 or I51C GFP-mSK1 were treated with either S1P (5 µM) or PMA (1 µM) or carbachol (100 µM) for 10 min. Cells were processed and mounted with DAPI to stain DNA (blue). (A) 40x oil magnification photomicrographs of GFP fluorescence in cells over-expressing WT, K49E or I51C GFP-mSK1. Results are representative of 3 independent experiments. Inset is a western blot probed with anti-GFP antibody showing the over-expression of GFP-mSK1 and the bar graph shows transfection efficiency (%). No significant difference between WT vs mutants (one way ANOVA with Tukey post hoc test). (B) The bar graphs represent the AUC (upper) of transfected GFP-mSK1 (WT or mutant) translocation (n=5) and the % of cells (lower) containing translocated GFP-mSK1 (WT or mutant) (n=3); *p<0.05, ***p<0.001 and ****p<0.0001 for K49E or I51C mutant vs WT for a given stimulus; *p<0.05, **p<0.01 and ***p<0.0001 for stimulus vs respective control for transfected WT GFP-mSK1 or K49E or I51C mutants (two way ANOVA with Tukey post hoc test). (C) Bar graphs representing the % cells containing GFP-mSK1 (WT or K49E or I51C) in lamellipodia and filopodia micro-domains in the PM in response to stimulus (n=4); ***p<0.001 and ****p<0.0001 for stimulus vs control for transfected WT GFP-mSK1 or K49E or I51C mutants (two way ANOVA with Tukey post hoc test).

Fig. 8 Duolink PLA for protein interaction detection between Myc-WT mSK1 and GFP-WT mSK1, GFP-K49E mSK1 or GFP-I51C mSK1 in MCF-7L cells. (A) MCF-7L cells over-expressing WT Myc-mSK1 with WT GFP-mSK1, K49E GFP-mSK1 or I51C GFP-mSK1 was confirmed by fluorescence
microscopy prior to Duolink PLA to assess protein interactions. PLA was carried out to assess differences in protein interaction between WT Myc-mSK1 and WT GFP-mSK1 or K49E GFP-mSK1 or I51C GFP-mSK1 when co-expressed. Untransfected cells (control) and cells expressing only WT GFP-mSK1 or WT Myc-mSK1 were used as negative controls. Cells were processed for PLA and mounted with DAPI to stain DNA (blue). (B) Quantitative analysis of the cellular PLA signals was carried out. The bar graphs represent the PLA signal mean gray value (mean ± SEM) with experiments carried out in triplicate (50 cells total per sample group shown) *** p<0.001 for WT Myc-mSK1/mutant GFP-mSK1 combination vs WT Myc-mSK1/WT GFP-mSK1 combination (one way ANOVA with Bonferroni post hoc test). (C) Representative PLA images are shown. Scale bar = 20 μm.

Fig. 9 Characterisation of C-terminal tail (T1-T5) mutants. MCF-7L cells separately over-expressing WT-GFP-mSK1 or each of the T1-T5 mutants were treated with S1P (5 μM) or PMA (1 μM) or carbachol (100 μM) for 10 min. Cells were processed and mounted with DAPI to stain DNA (blue). (A) Schematic to show the C-terminal amino acid sequence of the T1-T5 mSK1 mutants. (B) Western blot probed with anti-GFP antibody showing the over-expression of WT and T1-T5 GFP-mSK1; the bar graph shows transfection efficiency. Re-probing with GAPDH is used to confirm similar protein loading. No significant difference between WT v mutants (one way ANOVA with Tukey post hoc test). (C) 40x oil magnification photomicrographs of GFP fluorescence in cells separately over-expressing WT GFP-mSK1 or each of T1-T5 GFP-mSK1 mutants. Results are representative of 3 independent experiments. (D) The bar graphs represent the AUC of transfected GFP-mSK1 (WT or T1-T5) translocation (n=5) and the % cells containing translocated GFP-mSK1 (WT or T1-T5) at the PM in response to stimulus (n=3); * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 for AUC for stimulus vs control for each of WT or T1-T5 (unpaired t test).

Fig. 10 Time-course of SK1 translocation. MCF-7L cells over-expressing WT-GFP-mSK1 or T1 mutant were treated with and without carbachol (100 μM) for 1-10 min. Cells were processed and mounted with DAPI to stain DNA (blue). (A) 40x oil magnification photomicrographs of cells over-expressing WT GFP-mSK1 and T1 GFP-mSK1 detected with GFP. Representative results of 3 independent experiments. (B) The bar graphs represent the AUC of transfected WT GFP-mSK1 or T1 mutant translocation (n=5); * p<0.05, ** p<0.01 and **** p<0.0001 for treated vs control (unpaired t test).

Fig. 11 S1P levels. MCF-7L cells were transfected with WT GFP-mSK1 or T1-T5 GFP-mSK1 mutants or K49E GFP-mSK1 mutant or I51C GFP-mSK1 mutant and S1P levels measured by LC-MS/MS. * p<0.05, ** p<0.01 and *** p<0.001 for SK1 (WT or mutant) transfected vs GFP transfected (unpaired t test); +++ p<0.0001 for T5 mutant vs WT SK1 transfected (unpaired t test) for triplicate samples.
Fig. 12 Hypothesised structural rationale for alignment of membrane engagement determinants orchestrated by R-loop and C-terminal tail. SK1 exhibits a long, twisted strand pair (green ribbon) that acts as a ‘connecting rod’ between the NTD (slate-blue ribbon) and CTD (salmon ribbon), illustrated here from the crystal structure of hSK1 (PDB: 4L02). Flex and twist about the connecting rod is postulated to control inter-domain movement and thence topographical alignment or misalignment of key membrane-interfacing determinants—K27, K29, R186, LBL-1 hydrophobic surface (obscured in this view perspective)–––for anionic phospholipid-enriched membrane engagement. The protein conformation exhibited in this crystal structure, where the C-terminal tail (red dotted line) has been removed after G364, likely corresponds to the active state alignment of the NTD and CTD and features inter-domain hydrogen bonding from N89 and engagement of connecting rod histidines (H156/H355) by R-loop D235 (labelled) to stabilise the observed conformational arrangement of domains. In cytosol, with the intact C-terminal tail, a ‘locking sequence’ (residues 6-10 preceding the C-terminus in mSK1) is postulated to obstruct adoption of the active state conformation and maintain the NTD and CTD membrane-interfacing determinants in a misaligned state. This may involve folding of the locking sequence onto the CTD (blue dotted line), potentially with rotation about the twisted pair. G_q signaling manipulates a ‘key sequence’ (PPEEP) at the C-terminus, possibly by binding of an adapter protein, to remove the influence of the locking sequence and allow adoption of the conformation with aligned membrane-interfacing determinants. R-loop phosphorylation on S225 (labelled) by ERK also drives adoption of an active state conformation in a manner that does not require the presence of the C-terminal key. In principle, this may involve a structural transition in the R-loop that overturns the hypothesised folding of the locking sequence across the CTD and forces an active-state twist on the connecting rod. Alternatively, R-loop phosphorylation might facilitate protein recruitment to stabilise the active-state conformation and overturn the locking sequence.
