Supplementary Information

Materials and Methods.

Immune complex kinase assays. V-H69 and -H69 cells in SFM were washed three times in RPMI 1640, and 2 x 106 cell aliquots were incubated in this medium for 30 min at 37oC. Cells were treated in the presence or absence of 40 μM (TI-TITAT, (TI-TITAT or TITAT peptide for 4 h as indicated, prior to performing an immune complex kinase assay for S6K1 or S6K2 as described (Pardo et al., 2001). In separate experiments, HEK293Tet cells expressing tetracycline-inducible kinase active cytoplasmic forms of S6K1 or S6K2 were incubated with or without tetracycline prior to lysis and immune complex kinase assay.

In vitro kinase assay. 0.5 μg recombinant His-S6K and 4 μg recombinant PKCε were incubated on ice in 50mM TRIS (pH 7.5), 100 mM NaCl, 0.1 mM EDTA and 0.1% TritonX-100, 0.3 (v/v)% β-mercatptoethanol and 1 mM Na3VO4 in the presence or absence of recombinant active V600EB-Raf. The reaction was started by adding an ATP-mix resulting in a final concentration of 100 μM ATP, 10mM MgCl2 with or without 33 nCi/μl [γ-^{32}P]ATP and incubated for 30 min at 30ºC. As a positive control recombinant GST-MEK was used as a substrate for V600EB-Raf. Reactions were terminated in SDS-sample buffer and analysed by SDS-PAGE and autoradiography.

RNAi sequences. RNAi-mediated downregulation of PKCα, PKCε, S6K1, S6K2, B-Raf and Raf-1 was achieved using short-hairpin sequences cloned into pSUPER Retro constructs or oligonucleotide siRNA and are listed in the supplementary data. For pSUPER Retro-mediated downregulation, each protein was simultaneously targeted using three different short-hairpin sequences. Sequences were as follow: PKCα, ,CAAGGCTTCCAGTGCCAAG, GGAACACATGATGGATGGA, CATGGAACTCAGGCAGAAA; PKCε, TCTGCGAGGCCGTGAGCTT, CTACAAGGTCCCTACCTTC, GGAAGGGATTCTGAATGGT; S6K1, GGTTCTGGGCCAGGGATCC, GCTCTATCTCATTCTGGAC, CATCATCACTCTGAAAGAT; S6K2, GGGGGGCTATGGCAAGGTG, CGGAATCCCAGCCAGCGGA, GATACGGCCTGCTTCTACC; B-Raf, CAACAGTTATTGGAATCTC, CCTATCGTTAGAGTCTTCC, GAATTGGATCTGGATCATT; Raf-1, CAGTGGTCAATGTGCGAAA, GAACTTCAAGTAGATTTCC, CATCAGACAACTCTTATTG. Oligonucleotide siRNA against S6K2 and S6K1 were purchased from Dharmacon as SMARTpools.

Sequences were as follow: S6K2, GCAAGGAGUCUAUCCAUGAUU, GACGUGAGCCAGUUUGAUAUU, GGAAGAAAACCAUGGAUAAUU, GGAACAUUCUAGAGUCAGUUU; AS, 5'-PACUGACUCUAGAAUGUUCCUU; S6K1, GCAGGAGUGUUUGACAUAG, GACAAAAUCCUCAAAUGUA, CAUGGAACAUUGUGAGAAA, CCAAGGUCAUGUGAAACUA. Oligonucleotide targeting of B-Raf was achieved using a single sequence: AAAGAAUUGGAUCUGGAUCAU.

Reagents. Etoposide was purchased from Calbiochem. PKCα, PKCβ, PKCδ, PKCλ, PKCγ, PKCζ, PKCα, PKCε, Bcl-X_L and XIAP antibodies were purchased from Becton Dickinson. The phospho-PKCα and phosphor-S6 protein antibody was from Cell Signalling. The phospho-PKCε antibody against Ser729 and an additional PKCε antibody (for Western-blotting only) were obtained from Upstate. The phospho-PKCε antibody against Thr566 was as described previously (Parekh et al., 1999). S6K1, B-Raf, Raf-1, Lamin B and Actin antibodies were purchased from Santa Cruz. The S6K2 antibody was as described previously (Gout et al., 1998). Protein A and G were obtained from Amersham. Lipofectin, Lipofectamin Plus, G418, zeocin and puromycin were obtained from Invitrogen. The activated ERK antibody, etoposide, polybrene and crystal violet were obtained from Sigma. FGF-2, PD098059, Gö6976, Hispidin, BAPTA, Rottlerin and GF109203X were purchased from Calbiochem.

Supplimentary Figure Legends

Supplementary Figure 1: (A, B, C and D) PKCε controls FGF-2-mediated Erk phosphorylation in SCLC cells. (A) H510 cells were treated with or without a dose range of GF109203X (GF), Gö6976 (Go), Hispidin (His), BAPTA (BA) or Rottlerin (Rot) for 1 h prior to stimulation for 5 min in the presence or absence of either FGF-2 (0.1 ng/ml) or PDBu (400nM). Cell lysates were analysed by SDS-PAGE/Western-blotting for biphospho-ERK. Lamin immunodetection was used as loading control.
(B-top panel) Equal protein amounts from SCLC cell lines were compared for their PKC expression pattern. (B-bottom panel) SCLC cells in SFM were stimulated with and without FGF-2 for 5 min and cell lysates analysed by SDS-PAGE/WB for Erk phosphorylation. PKCε and PKCα levels (ODs from left panel) were compared to the ability to phosphorylate Erk in these SCLC cell lines. Results shown are representative of at least three independent experiments.

Supplementary Figure 2: PKCε forms a multiprotein complex with B-Raf and S6K2 in H510 cells. (A and B) H510 cells in SFM were treated with FGF-2 for the times indicated. Cell lysates were subjected to immunoprecipitation with either S6K1 or 2 (A), B-Raf or Raf-1 (B) antibodies prior to SDS-PAGE/Western Blotting (WB) for S6K1 and 2, B-Raf, Raf-1 and PKCε. (A and B) Results shown are representative of at least three independent experiments.

Supplementary Figure 3: PKCε is required for B-Raf association with S6K2. (A) Efficacy of single siRNAi oligonucleotide sequences (1 and 2) and Smartpools (P) directed against S6K1, S6K2, PKCe, PKCa, B-Raf and Raf-1. HEK293 cells were transfected with the oligonucleotides and lysates analysed for target downregulation 48h later by SDS-PAGE/WB. (B, C and D) PKCα (α) and B-Raf (B) or PKCε (ε) were downregulated using pSR retroviral RNAi vectors in HEK293 cells and compared to empty vector only (V) transfected cells for their expression of the RNAi targets and their ability to phosphorylate Erk (B and C) or form the S6K2/PKCε/B-Raf multiprotein complex (D) in response to FGF-2. (A-D) Results shown are representative of at least three independent experiments.

Supplementary Figure 4: S6K2 kinase activity protects HEK293 cells from serum deprivation and induces expression of Bcl-X_L and XIAP. (A and B) HEK293 expressing tetracycline-inducible kinase-active S6K1 (1KA) or 2 (2KA) were treated with or without tetracycline for 6h (B) or the time indicated (A). (A) Cells were grown in the absence of FCS and a cell death time-course performed. Cell death was assesed microscopically by dertermining Trypan blue positivity. Results shown are averages ± SEM of triplicates. (C) HEK293 cells were treated for 1h with or without 25μM PD098059 prior to stimulation with FGF-2 for 4h. (B and C) Cell lysates were analysed by SDS-PAGE/WB for the levels of Bcl-X_L and XIAP. Actin was used as a loading control. Results shown are representative of at least three independent experiments.

Supplementary Figure 5: S6K2 and B-Raf but not S6K1 single siRNA sequences prevent FGF-2-mediated rescue of etoposide treated H510 cells. H510 cells grown in SFM were transfected with either of two siRNA single sequences (#1 and #2 as

shown in Fig 3A) targeting S6K1, S6K2 or B-Raf as indicated. Two non-targeting sequences (sc#1 and 2) were used as controls. Cells were pre-incubated for 4 h with FGF-2 (F) prior to etoposide (E) treatment. Cell death was determined microscopically by Trypan blue exclusion. Results shown are average ± SEM of triplicates and are representative of at least three independent experiments.

Supplimentary Figure 6: S6K2 staining correlates with chemoresistance in human SCLC biopsy material. Formalin fixed and paraffin embedded biopsies from 22 patients with SCLC and NSCLC at presentation were sectioned and immunostained using a mouse anti-S6K2 monoclonal antibody (provided by Prof Gout, UCL, London) and Envision detection system (DAKO). Specificity for the target protein was controlled for by using standard protocols including known positive (H510) and negative (Type II pneumocytes) samples, irrelevant antibody and competing S6K2. The pathologist (Dr Neil Sebire, Hammersmith Hospitals) was blinded to the clinical outcome data to avoid reporting bias. The study and on going collection of SCLC and NSCLC biopsy material has been reviewed and approved by our local ethics review board.

Upper Panel: strong S6K2 immunostaining seen in most cancer cells in a biopsy from a patient with chemoresistant tumour (original magnification x 100).

Middle Panel: focal areas of moderate S6K2 staining in a biopsy from a patient with partially chemoresistant disease (original magnification x 100). Our hypothesis would be that these positive cells survived initial chemotherapy and if we had biopsy data on relapse perhaps all the cells would be positive for S6K2.

Lower Panel: absence of S6K2 staining in a biopsy from a patient with chemosensitive disease (original magnification x 100).

These results were seen in 2 of 4 chemoresistant patients, 3/3 partially chemoresistant and 6/6 chemosensitive patients with SCLC. To substantiate these results, we also examined S6K2 staining levels in biopsies from several NSCLC patients. In one patient who was resistant to therapy the tumour was diffusely positive, three of four early relapsing patients, the tumours were focally positive for S6K2 staining whilst three of four chemosensitive tumours were negative. The combined results of staining in both SCLC and NSCLC biopsies are summarised in the adjoining table. Together, these results suggest that S6K2 protein expression levels in biopsies from patients with lung cancer correlates with chemoresistance.