Supplementary information

Supplemental Figures

Supplemental Figure 1

Figure S1 (related to Figure 1). AKT activation induces mesenchymal cell migration and invasion. A. EGF induces AKT (pS473) and ERK phosphorylation and enhances migration and invasion of SW684 cells (WB). PI3K/AKT inhibition (via LY294002) but not ERK inhibition (UO126) abrogates EGF-induced migration and
invasion (see Figure 1C in manuscript for similar results in SKLMS1 cells); B. ERK inhibition does not abrogate EGF-induced SKLMS1 cell migration and invasion in contrast to the significant effect of PI3K/AKT inhibitors on these processes (See Figure 1C in manuscript); C. STS cells (HT1080 and SKLMS1) express significantly higher constitutive levels of phosphorylated AKT as compared to normal mesenchymal cells (NHDF = normal human dermal fibroblast; HC-SMC = human smooth muscle cells; HDMEC = human dermal microvessel endothelial cells; HUVEC = human umbilical vein endothelial cells); D. Normal human dermal fibroblasts were transfected to overexpress wild type AKT, dominant negative AKT (AAA), and activated AKT (DD) as depicted in WB (see below data confirming high transfection efficiency in these normal cells). Overexpression of AKT-DD significantly increased the number of migrating and invading cells (p<0.001) (see below confirmation that transfected cells are actually the cells migrating/invading); E. Vimentin extracted from STS cells (SW684) but not from normal mesenchymal cells (NHDF) is recognized by PAS antibody suggesting constitutive AKT-induced vimentin phosphorylation in tumor cells; F. Overexpression of AKT1 and to a larger degree activated AKT1 (DD) in NHDF induces vimentin phosphorylation; in contrast inactive AKT1 (AAA) does not result in vimentin phosphorylation. [Graphs represent the average of three repeated experiments ±SEM; * depict statistically significant effects (p<0.05); pAKT WB in all Figures refer to pS473]
A. Based on the high levels of protein identified by western blotting after transfection (figure 1B, etc.) it can be estimated that the efficiency of this procedure in normal cells (during exponential growth phase) is high. To further prove this and to quantitatively determine transfection efficiency, we transfected NHDF and HC-SMC with a GFP containing plasmid and evaluated the number of GFP expressing cells using FACS analysis.

B. All migration and invasion experiments were conducted using control pcDNA transfected cells and target transfected cells. Differences in migration and invasion between control and target transfected cells were noted and this confirms that the transfection does modify these processes. These experiments followed commonly used methodologies and interpretation as per numerous other studies. To further validate this and as a proof of principle we have co-transfected HC-SMC with vimentin-GFP and AKT-DD-DsRed and used these cells for migration and invasion. As shown above, cells in the bottom of the membrane were found to express GFP/RFP, confirming that transfected cells are among the cells that have migrated/invaded.
Figure S2 (related to Figure 2). Primary human STS cell cultures and Functional effects of AKT isoforms. A. Upper panel: H+E staining of human tumors of which primary cultures were derived demonstrating the pleomorphic high grade nature of these specimens. All evaluated cell strains demonstrated anchorage independent growth in soft agar to varying levels (middle panel) and aneuploid karyotype based on gimsa staining. These experiments validate the tumor origin of the cells in culture. (HGS-446 = unclassified high grade pleomorphic sarcoma; HGS-396 = unclassified high grade pleomorphic sarcoma; Leio-177 = high grade leiomyosarcoma; MFH-M = high grade...
malignant fibrosarcoma; HGS-841 = unclassified high grade pleomorphic sarcoma); B. No phosphorylation of endogenous AKT2 can be observed in SKLMS1 cells. However, forced expression of AKT2 does result in its constitutive and inducible phosphorylation; C. AKT2 overexpression rescues the inhibitory effects of AKT1 knockdown on SKLMS1 cells; D. Over-expression of activated AKT2 enhances SKLMS1 cell migration and invasion. [Graphs represent the average of three repeated experiments ±SEM; * depict statistically significant effects (p<0.05); pAKT WB in all Figures refer to pS473]
Figure S3 (related to Figure 3). AKT1:vimentin binding is observed in STS cells. A. Double immunoflorescence staining (using anti vimentin and anti AKT1 antibodies) suggests AKT1:vimentin cytoplasmic colocalization (especially surrounding the nucleus) in SKLMS1 cells; B. DsRed-AKT1 and Vim-GFP co-transfection into SKLMS1 cells demonstrate protein colocalization in live cells; EGF stimulation does not affect protein co-localization; C. protein-fragment complementation assay (RePCA): bait protein (AKT1) was fused to one fragment of a rationally dissected venous fluroscent protein; the second complementary fragment of the fluroscent protein was fused to vimentin in reading frame. Protein expression was confirmed by WB. Protein-protein interaction was demonstrated by positive florescent expression as was evaluated by microscopy and flow cytometry. VC-PDK1 (known to bind AKT1) was used as a positive control; D.
HEK293T cells were transfected to transiently express either vimentin or both vimentin and HA-tagged AKT1. WB confirmed the expression of the relevant proteins (upper panel). Anti-HA IP in these cells resulted in vimentin pull-down in HA-AKT/vimentin cotransfected cells (middle panel). Reverse IP for vimentin demonstrated HA-AKT1 pull-down in cells expressing both AKT1 and vimentin (lower panel); E. Similar to data shown above (D.) AKT1:vimentin binding was seen in MCF7 cells transfected to transiently express either vimentin or both vimentin and AKT1. F. A co-sedimentation assay (see Supplemental M+M for details) was conducted to evaluate the association of AKT1 with polymerized vimentin. The data above suggests that AKT1 co-sediments in the presence of polymerized vimentin but not alone.
Figure S4 (Related to Figure 5). Vimentin expression and possible phosphorylation in STS; a possible novel AKT1 substrate. A. H+E (upper panel) and
vimentin (lower panel) of selected human high grade STS specimens used for experiments depicted in Figure 5A in manuscript demonstrating cytoplasmic vimentin expression; B. Double immunoflorescence depicting colocalization of vimentin and PAS in SKLMS1 cells. This colocalization was abrogated after short treatment (4h) with LY294002; C. Total cell lysates of SKLMS1 cells, untreated or treated with LY294002 or A563 (not shown) for 2 hours, were subjected to iso-electric focusing using pH strips between 3 and 10 pl units, and between 4 and 7 pl units. The second dimension was performed with 4%-16% polyacrylamide gels and the proteins were transferred to PVDF membranes for Western blot analysis, using a specific anti-vimentin antibody to visualize shifts in vimentin in the pl axis. A shift in vimentin pH (double headed arrow) after AKT inhibition was identified. Furthermore, increased basic smear to the vimentin protein train can be seen (blue circles). These findings suggest significant changes in vimentin posttranslational modifications (most likely related to changes in phosphorylation state) induced by AKT inhibition. Interestingly spots of lower molecular weight (small arrows) can be identified after LY294002 treatment suggesting possible treatment-induced vimentin degradation; D. MALDI TOF-TOF scans of tripsinized vimentin samples. MS zoom scan of the vimentin+AKT1 sample from 1450.0 to 1700.0 Da. demonstrated a 1575.78 singly charged peptide corresponding to the TYpSLGSALRPSTSR peptide (black arrow); E. In contrast, the MS zoom scan of vimentin without AKT1 treatment demonstrated that the 1575.78 singly charged peptide is not detected even though the overall peak intensities are greater than in the AKT1 treated sample. F. MRM analysis of AKT treated recombinant vimentin. The main panel displays the peak intensity of the eluted TYpSLGSALRPSTSR phosphopeptide (green signal trace). The inset reveals the intensity of one of the parent peptide (788.4 Da m/z) fragment ion transitions (739 .4 Da m/z). In the presence of activated AKT1 the abundance of phospho-vim S39 peptide is ~10³ times as compared to the non-phosphorylated peptide.; G. MRM analysis of AKT-
untreated recombinant vimentin. The main panel displays the peak intensity of the
TYPpSLGSALRPSTSR phosphopeptide (green signal trace), as measured by the mass
spectrometer as it comes off of the column. The inset reveals the intensity of one of the
parent peptide (788.4 Da m/z) fragment ion transitions (739.4 Da m/z). The intensity of
the phosphorylated peptide peak is approximately 450-fold less intense than the AKT-
treated sample (see panel C.); many more background peaks are seen here because
the TYPpSLGSALRPSTSR signal intensity is within the noise of the assay.
**Supplemental Figure 5**

**Figure S5 (related to Figure 7).**

Shown are enlarged images of Figure 7F panels to enable to more clearly demonstrate the filamentous structure of vimentin after WT, S39A, and S39D transfection. All three vimentin proteins (even when GFP tagged) harbor the capacity to assemble into filamentous structures. These findings support previous published data (Eriksson JE, He T, Trejo-Skalli AV, Härmälä-Braskén AS, Hellman J, Chou YH, Goldman RD. J Cell Science [2003] 117, 919-932). It has been shown that over expression of WT and unphosphorylable vimentin have no significant effect on the incorporation rates of vimentin subunits into assembled polymers while phosphorylated vimentin has significant impact on filament formation dynamics, decelerating the process – however, three hours after forced protein expression all three vimentin proteins result in fully assembled filamentous vimentin, as shown also by our data.
Table S1 (related to Figure 3). AKT1-Immunoprecipitated Proteins Identified by Mass Spectrometry

| Protein Band\(^a\) | Protein Name             | SwissProt Accession No. | Peptide Match | Protein Score | \(M_r\) (kDa)\(^b\) |
|---------------------|--------------------------|-------------------------|---------------|---------------|---------------------|
| 1                   | Tropomyosin 4            | Gi4507651               | 14            | 405           | 28                  |
| 2                   | Tropomyosin 2            | Gi47519616              | 19            | 401           | 33                  |
| 3                   | \(\beta\)-Actin\(^d\)   | Gi126338084             | 13            | 809           | 42                  |
| 4                   | \(\beta\)-Tubulin\(^d\) | P68371                  | 3             | 20            | 49                  |
| 5\(^c\)             | Vimentin                 | P08670                  | 19            | 190           | 54                  |
| 6\(^c\)             | AKT                      | P31749                  | 8             | 80            | 56                  |
| 7\(^c\)             | DDX5                     | P17844                  | 3             | 20            | 63                  |
| 8\(^c\)             | GRP75                    | P38646                  | 4             | 40            | 73                  |
| 9                   | TRH-degrading enzyme     | Gi1486898               | 5             | 51            | 84                  |
| 10                  | \(\alpha\)-Actin         | Gi73947744              | 3             | 100           | 108                 |

\(^a\)Bands were excised from Coomassie-stained SDS-PAGE and identified by mass spectrometry.

\(^b\)\(M_r\) was calculated from primary structure and correlates favorably with band size observed in SDS-PAGE (Figure 3A).

\(^c\)Proteins 5, 6, 7, and 8 were confirmed by western blotting (Figure 3A).

\(^d\)Marked proteins were previously identified as putative AKT binding partners in epithelial cancer cells (MCF-7; Vandermoere F, El Yazidi-Belkoura I, Demont Y, Slomianny C, Antol J, Lemoine J, Hondermarck H. (2007) Mol Cell Proteomics. 6, 114-24). \(\beta\)-Actin was further shown to be a potential substrate for AKT kinase activity.
Table S2 (related to Figure 5): Mascot search results of the MS/MS Fragmentation of the AKT + ATP-treated vimentin phosphopeptide $^3$TYSLSALRPSTSR50 showing neutral loses

|   | A       | a**    | a'     | a***   | b   | b**    | b*     | b***   | Se q. | y       | y**    | y*     | y***   | #  |
|---|---------|--------|--------|--------|-----|--------|--------|--------|-------|---------|--------|--------|--------|----|
| 1 | 74.0600 | 37.5337|        |        | 102.0550 | 51.5311|        |       | T     |         |        |        |        | 14 |
| 2 | 237.1234| 119.0653|        |        | 265.1183 | 133.0628|        |       | Y     | 1376.7281 | 668.8677 | 1359.7015 | 680.3544 | 13 |
| 3 | 306.1448| 153.5761|        |        | 334.1397 | 167.5735|        |       | S     | 213.6648 | 607.3360 | 1196.6382 | 598.8227 | 12 |
| 4 | 419.2289| 210.1181|        |        | 447.2238 | 224.1155|        |       | L     | 144.6433 | 572.8253 | 1127.6187 | 584.3120 | 11 |
| 5 | 476.5204| 238.6288|        |        | 504.2463 | 252.6263|        |       | G     | 1031.5592 | 516.2833 | 1014.5327 | 507.7700 | 10 |
| 6 | 562.2824| 282.1448|        |        | 591.2773 | 296.1423|        |       | S     | 974.5378 | 487.7725 | 957.5112 | 479.2592 |  9 |
| 7 | 634.3195| 317.6634|        |        | 662.3144 | 331.6608|        |       | A     | 887.5057 | 444.2565 | 870.4792 | 435.7432 |  8 |
| 8 | 747.4036| 374.2054|        |        | 775.3985 | 388.2029|        |       | L     | 816.4686 | 408.7380 | 799.4421 | 400.2247 |  7 |
| 9 | 903.5047| 452.2560|        |        | 931.4996 | 466.2534 | 914.4730 | 457.7402 |       | 703.3846 | 352.1959 | 686.3580 | 343.6826 |  6 |
| 10|1000.5974| 500.7824|        |        | 1028.5520 | 514.7798 | 1011.5258 | 508.2665 |       | 547.2835 | 274.1454 | 530.2569 | 265.6321 |  5 |
| 11|1087.5895| 544.2984|        |        | 1115.5841 | 558.2958 | 1098.5578 | 549.7826 |       | 450.2307 | 225.6190 | 433.2041 | 217.1057 |  4 |
| 12|1188.6371| 594.8222|        |        | 1216.6321 | 608.8197 | 1199.6065 | 600.3064 |       | 363.1987 | 182.1030 | 348.1721 | 173.5897 |  3 |
| 13|1275.6692| 638.3382|        |        | 1303.6649 | 652.3357 | 1286.6375 | 643.8224 |       | 262.1510 | 131.5791 | 245.1244 | 123.0659 |  2 |
| 14|1363.7371| 683.3811|        |        | 1391.7331 | 697.3361 | 1374.7086 | 687.3244 |       | 175.1190 | 88.0631  | 158.0924 | 79.5490  |  1 |

* Matched peptides are highlighted in red
Table S3 (related to Figure 5). Vimentin Motif Scan Analysis for Potential AKT Phosphorylation Sites

| Site | Score | Percentile | Sequence |
|------|-------|------------|----------|
| Ser39 | 0.4039 | 0.049% | TTSTRYS*LGSALRP |
| Thr33 | 0.7142 | 3.928% | SSRSYVTTS*TRTYSL |
| Ser325 | 0.6829 | 2.818% | EYRRQVQS*LTCVEVA |

* predicted AKT phosphorylation sites in human vimentin by Motif Scan

| Gene symbol            | sequence     | AKT phosphorylation site |
|-----------------------|--------------|--------------------------|
| VIM (human)           | TTSTRTYSLGSALRP | Ser39*                  |
| VIM (mouse)           | TTSTRTYSLGSALRP | Ser39*                  |
| VIM (rat)             | TTSTRTYSLGSALRP | Ser39*                  |
| 14-3-3 zeta (human)   | VVGARRSSWRVSSI | Ser58**                  |
| Bim (human)           | FIFMRSSLLSSRSSS | Ser87**                 |
| CTNNB1 (human)        | QDTRQRTSMGGTQQQ | Ser552**                |
| eNOS (human)          | SYKIRFNSISCSDPL | Ser614**                |
| IRS1 (human)          | VPSGRKGSGDYMPMS | Ser629**                |
| P48phox (human)       | QDAYRRNSVRFLQQR | Ser328**                |

* Vimentin Ser39 and flank sequence are highly conserved in human, mouse and rat

** Confirmed to be AKT phosphorylation sites *in vivo and in vitro*

AKT1 phosphorylation motif: **RXRXXS/T or RXXS/T**
Table S4 (related to Figure 6): *in vivo* experiments to determine the effects of vimentin phosphorylation mutants on STS local and metastatic growth

| Cells                  | Tumorigenicity* | Average Tumor Volume** (±SD) | P Value*** | Lung Metastasis**** | P Value*** |
|------------------------|-----------------|------------------------------|------------|---------------------|------------|
| SKLMS1-VIMS39A/GFP     | 5/5             | 626mm$^3$ (±56)              | --         | 0/5                 | --         |
| SKLMS1-VIMS39D/GFP     | 5/5             | 1288mm$^3$ (±55)             | 0.0007     | 4/5                 | 0.016      |

*Number of tumors versus number of SCID mice injected
**Average tumor volume at six week post inoculation
***P values were determined by comparing the two mice cohorts using a Student t-test
**** Macroscopic lung metastasis development versus number of SCID mice injected (tail-vein injection)
Supplementary experimental procedures

Cell Lines and Primary Human STS Cell Cultures

The following complex-karyotype human STS cell lines were used: SKLMS1 leiomyosarcoma (purchased from the American Type Culture Collection [ATCC]), HT1080 and SW684 fibrosarcoma (ATCC), A204 unclassified sarcoma (ATCC) maintained in McCoy's 5A, and MPNST724 malignant peripheral nerve sheath tumor (a kind gift from Dr Jonathan Fletcher, Brigham & Women's Hospital). The breast cancer cell lines MCF7 and MDA231 (ATCC) and the human embryonic kidney cell line HEK293 (ATCC) were also used. Primary normal mesenchymal cell cultures included normal human dermal fibroblasts (NHDF; PromoCell, Heidelberg, Germany) maintained in fibroblast growth medium (PromoCell, Heidelberg, Germany, cat. No. C-23010), normal human smooth muscle cells HC-SMC and HA-SMC (ScienCell, Carlsbad, CA) maintained in smooth muscle cell medium (Cat No.1101, ScienCell, Carlsbad, CA), and human endothelial cells HUVEC and HDMEC (Cambrex, Walkersville, MD) maintained in EGM-2 Endothelial Growth Media (Cat. No. CC-4176, Lonza, Walkerville, MD).

Human STS primary cultures were isolated as described below. All cell lines, except as specifically indicated above, were maintained in DMEM supplemented with 10% fatal bovine serum (FBS).

STS Primary Culture Isolation and Characterization

These procedures were conducted with approval from the Institutional Review Board at The University of Texas M. D. Anderson Cancer Center and informed consent from patients. Tumor cell isolation was conducted as previously described (footnote). Briefly, fresh sterile samples from surgically resected tumors were minced in culture medium and then digested via incubation with collagenase type I (3%), DNase I (0.02%), and hyaluronidase (1.5 mg/ml) at 37°C for 2-4 h. The sample was strained through a wire
mesh screen, and undigested tissue was discarded. After centrifugation, washes, and
resuspension in PBS, the sample was gently transferred to Histopaque tubes containing
10 ml Histopaque (100%; Sigma) overlayed with 15 ml of Histopaque (75%). The tubes
were then centrifuged at 40°C for 30 min at 1200g. After centrifugation, the tumor cells
located in the top interface (over the 75% Ficoll were collected and plated. Cells were
cultured and passaged in DMEM containing 10% FBS (Life Technologies), penicillin-
streptomycin, and bovine pituitary extract. All primary cultures exhibited a typical spindle-
shaped morphology. For all experiments, cells passaged fewer than five times were
used. All cells were routinely examined with GenProbe (Fisher Scientific) and found to
be negative for mycoplasma contamination.

**Chromosome Analysis**

Subconfluent sarcoma cell cultures were treated with 15 ml of a solution containing 10
μg/ml colcemid (Life Technologies) for 2 h at 37°C. The cells were harvested after
treatment with a solution containing 0.25% trypsin for 1 min. Pooled cells were then
resuspended in a hypotonic solution containing 0.075 M KCl; 1 ml of fixation solution
(75% methanol, 25% glacial acetic acid) was added, mixed via inversion, and
centrifuged. The pellet was resuspended in 5 ml of fixation solution, washed several
times, and dropped onto slides. The slides were stained with Giemsa stain (Biomedical
Specialties), and metaphase chromosomes were analyzed and counted under a light
microscope (100x oil lens) as previously described¹. Images were captured
using a SPOT digital camera (Diagnostic Instruments).

¹ Hu M, Nicolson GL, Trent JC 2nd, Yu D, Zhang L, Lang A, Killary A, Ellis LM, Bucana CD,
Pollock RE (2002) Cancer 2002, 95, 1569-76.
**Soft Agar Colony Formation Assay**

The soft agar colony formation assay was performed as previously described\(^1\). Briefly, 0.5 ml of an underlayer consisting of Dulbecco's modified essential (DME) F-12 medium, 10% FBS, and 0.7% agarose was plated in 24-well plates. Two thousand cells per ml were suspended in DME/F-12 medium with 10% FBS and 0.35% agarose, and 0.5 ml (1,000 cells/well) of the suspension was plated on top of the gelled underlayer. After incubation in 5% CO\(_2\) and 95% air at 37°C for 3 weeks, cell colonies were stained with iodonitrotetrazolium violet (Sigma) and counted under a light microscope.

**Reagents**

Commercially available antibodies were used to detect AKT, AKT1, AKT2, AKT3, Ser473-pAkt, PAS (phospho-AKT substrate), phospho-GSK3, activated caspase-3, pERK1/2, phosphor-p70S6K, p70S6K, and Myc-tag (Cell Signaling); vimentin (V9), GSK3, GRP75 (D9), MDM2, GFP, GST, ERK1/2, and \(\beta\)-actin (Santa Cruz Biotechnology); DDX5 (Abcam); and HA (HA.11 clone 16B12, Covance Research Products).

Human EGF and HGF were purchased from R&D Systems. Estradiol (0.72 mg of 17\(\beta\)-estradiol) 60 days slow-release pellets were purchased from Innovative Research of America (Toledo, OH). The specific AKT kinase inhibitor A674563 (A563) was a kind gift from Abbott Laboratories. The PI3K inhibitor LY294002, the MEK inhibitor UO126, and the mTORC1 inhibitor rapamycin were purchased from Cayman Chemical (Ann Arbor, MI); the proteosome inhibitor was purchased from Assay Designs (Ann Arbor, MI); and the caspase inhibitor Z-VAD was purchased from Promega. Constitutively activated human AKT1, AKT2 and recombinant human vimentin were purchased from Invitrogen. Protein G Sepharose beads were purchased from GE Healthcare.
**Constructs and Protein Purification**

DNA plasmids encoding HA-tagged human wild-type AKT1, AKT2, AKT1-DD (harboring activating mutations T308D and S473D, thus making it constitutively active), AKT2-DD (constitutively active), AKT1-AAA (a mutant in which both the activating phosphorylation sites and the ATP-binding lysine of AKT are mutated to alanine [K179A, T308A, S473A], rendering this mutant dominant negative) and DsRed2-AKT1 were gifts from Dr. Gordon Mills. The HA tag was placed at the N terminus, upstream of the PH domain.

Wild-type human vimentin cDNA (pcDNA3-VIM) was a gift from Dr. Vincent Cryns (Northwestern University). Site-directed mutagenesis was performed according to the manufacturer’s protocol (Stratagene) to replace vimentin Ser39 with alanine (pcDNA3-VIMS39A) using the primers 5′-CACCCGCACCTACGCCCTGGGCAGCGCG-3′ (sense) and 5′-CGCGCTGCCAGGGCGTAGGTGCAGGGGTG-3′ (antisense) or with aspartic acid (pcDNA3-VIMS39D) using the primers 5′-CACCCGCACCTACGCCCTGGGCAGCGCG-3′ (sense) and 5′-CGCGCTGCCAGGGCGTAGGTGCAGGGGTG-3′ (antisense). Site-directed mutagenesis was also used to replace vimentin Ser325 with alanine (pcDNA3-VIMS325A) using primers 5′-GGAGACAGGTGCAGGCCCTCACCTGTGAA-3′ (sense) and 5′-TTCACAGGTAGGGCCTGCACCTGTCTCC-3′ (antisense).

To generate constructs for vimentin-GFP fusion protein (pEGFPN1-VIM), vimentin cDNA fragments were amplified by PCR with specific primers 5′-CCCAAGCTTATGTCCACCAGGTCCGTC-3′ (N-terminal) and 5′-CGGGATCCTTCAAGGTCATCGTGATGCTG-3′ (C-terminal) and cloned into HindIII and BamHI sites of pEGFPN1. To generate pEGFPN1-VIMS39A and pEGFPN1-VIMS39D, we used the same primers as we used to generate VIMS39A and VIMS39D in pcDNA3.
To generate constructs for bacterial expression of full-length or truncated AKT1 and vimentin functional domains, the following primers were used to amplify cDNA fragments: pGEX4T1-VIM-FL, 5'-CGGGATCCATGTCACCAGGTGCTCTTCATCGTG-3' (N-terminal) and 5'-CGGAATTCTATTCAAGGTCATCGTG-3' (C-terminal); pGEX4T1-VIM-HEAD, 5'-CGGGATCCATGTCACCAGGTGCTCTTCATCGTG-3' (N-terminal) and 5'-CCGGAATTCTAGGGTGCGGTTCTTTGAACCT-3' (C-terminal); pGEX4T1-VIM-CC, 5'-CGGGATCCACGACAAGGTGGAGCTGCAG-3' (N-terminal) and 5'-CCGGAATTCTACCTGCTCTCCTCGCCTTCCAG-3' (C-terminal); pGEX4T1-VIM-TAIL, 5'-CGGGATCCATTTCTCTCTGCTCTCCTTTCAAC-3' (N-terminal) and 5'-CCGGAATTCTATTCAAGGTCATCGTG-3' (C-terminal); pGEX4T1-AKT-FL, 5'-CGGGATCCATGAGCGACGTGGCTATTGTG-3' (N-terminal) and 5'-CCGGAATTCTAGGGTGCGGCTCCATCGTG-3' (C-terminal); pGEX4T-AKT1-PH, 5'-CGGGATCCATGAGCGACGTGGCTCCATCGTG-3' (N-terminal) and 5'-CCGGAATTCTACCTGCTCTCCTCGCCTTCCAG-3' (C-terminal); pGEX4T1-AKT1-CAT, 5'-CGGGATCCGACGGCCTCAAGAAGCAGGAG-3' (N-terminal) and 5'-CCGGAATTCTAAAGAAGCGATGCTGCATGAT-3' (C-terminal); pGEX4T1-AKT1-TAIL, 5'-CGGGATCCGCCGGTATCGTGTGGCAGCAC-3' (N-terminal) and 5'-CCGGAATTCTAGGGTGCGGCTCCATCGTG-3' (C-terminal). The PCR products were gel–purified, digested with relevant restriction endonucleases, and cloned into BamHI and EcoRI sites of pGEX4T1 (GE Healthcare). GST fusion proteins were inducibly expressed in *Escherichia coli* strain BL21 and purified by glutathione-Sepharose chromatography (GE Healthcare). GST fusion proteins were subjected to SDS-PAGE followed by staining with Coomassie blue. All constructs were confirmed by DNA sequencing.
Expression of GST and GST fusion proteins was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.2 mM) using pGEX-4TL plasmid. For large-scale batch purification of GST fusion proteins, an overnight culture was set up in 50 ml of LB medium supplemented by 100 μg/ml ampicillin. Five milliliters of the overnight culture were seeded in 500 ml of LB with 100 μg/ml ampicillin and grown in a 37°C shaker incubator until absorbance at 600 nm was between 0.5 and 0.8. GST fusion protein expression induction was done by adding IPTG (final concentration of 0.2 mM), culturing in a 37°C shaker incubator for 3 h, and then transferring into Sorvall tubes (250 ml). Cells were pelleted by centrifuging at 3,000 g for 10 min at 4°C. Medium was decanted and cells resuspended in 30 ml of ice-cold TNE (10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA), followed by centrifugation at 12,000g for 10 min in a Sorvall centrifuge at 4°C. Supernatant was transferred to a 50 ml tube, and 2 ml of a 50% slurry of glutathione-Sepharose-4B beads (GE Healthcare) was added. After incubation at room temperature in the rotator for 30 min, the sample was spun down at 3,000 g at 4°C for 1 min. Supernatant was removed, and beads were washed eight times with 1x PBS containing 1% Triton X-100. After final washing, beads were resuspended with 1 ml of 1x PBS to make 2 ml of a 50% slurry. 10 μl of the 50% slurry were used for protein concentration determination.

To generate activated AKT1 and kinase dead AKT1 proteins for in vitro kinase assays HA-tagged AKT1DD and AKT1AAA were transfected into 293 cells using lipofectamine 2000. Forty eight hours post-transfection, cells were harvested and total protein was extracted; protein G beads conjugated anti-HA antibody was used to immunoprecipitate HA-AKT1DD and HA-AKT1AAA. HA-AKT1DD and HA-AKT1AAA proteins were eluted from beads by adding 60 μL of 0.2 M Glycine (pH 2.5) and mixing to resuspend the beads, then incubating for 1–2 minutes at room temperature. Supernatant was transferred into a new tube and neutralized by adding 5 μL of 1 M Tris
(pH 8.5). Bradford protein assay was used to determine protein concentration. 1μg of AKT1DD or AKT1AAA protein was used for in vitro kinase assay.

Morpholino oligomers targeting the splicing junction between the first exon and second intron of human vimentin pre-mRNA (sequence, TTGCATGGGCGCAGCCTTACTTTC) were purchased from Gene Tools. Two different sets of siRNA construct pools targeting AKT1 (Dharmacon: On-TARGET plus SMARTpool cat. no. L-003000-00; Santa Cruz: sc-29195), AKT2 (Dharmacon: On-TARGET plus SMARTpool cat. no. L-003001-00; Santa Cruz: sc-29197), and vimentin (Dharmacon: On-TARGET plus SMARTpool cat. no. L-003551-00; Santa Cruz: sc-29522) as well as non-targeting siRNA were utilized.

**Transfection Procedures**

Plasmid DNA and siRNA were introduced into cells using Lipofectamine 2000 (Invitrogen) per manufacturer instructions. Briefly, 2x10^5 cells were plated in each well of a six-well plate and incubated overnight. Cells were then incubated with a mixture of plasmid DNA (2μg) or siRNA (20 nM) and Lipofectamine 2000 (10 μl) diluted in Dulbecco’s modified Eagle medium (DMEM) for 24 h, followed by incubation in regular medium. Cells were harvested at indicated time points for specific experiments.

Anti-vimentin morpholino oligos and standard non-targeting control morpholino oligos were delivered into cells by Endo-Porter delivery reagent per manufacturer’s protocol (Gene Tools). Briefly, 3x10^5 cells plated in each well of a six-well plate were incubated overnight with regular culture medium. The medium was then replaced with fresh medium containing morpholino oligos (10 μM). After thorough mixing, Endo-Porter reagent (12 μl) was added. Cells were harvested for further studies at indicated time points.
**Co-sedimentation assay**

Human vimentin (20µg from R&D, cat#2105-VI) was incubated in polymerization buffer (5mM PIPES, 1mM DTT, and 150mM NaCl) to form polymerized vimentin, which was further incubated with AKT1 (2µg from Invitrogen, cat#P2999) for 16 hours at 4°C and subjected to ultracentrifugation at 100,000xg for 30min. The supernatant and pellet fractions were analyzed by western blot for AKT1 and vimentin.

**Fluorescence Microscopy**

STS cells transfected to express relevant proteins as described in text and Figure Legends were grown to approximately 60% confluence in eight-well Lab-Tek chamber slides (Thermo Fisher Scientific); when needed each of these cells was then treated as indicated. Similarly, STS cells grown in chamber slides were subjected to co-immunoflorescent staining with indicated primary and secondary antibodies. Slides were washed and fixed with 4% paraformaldehyde. Nuclei were counterstained with Hoechst 33258 (1:100,000; Sigma-Aldrich) for 10 min, washed twice with 1x PBS, and sealed by cover slides using an anti-fade immunofluorescence mounting solution. Fluorescence expression was evaluated using a Leica DM4000B microscope and a Leica HCxPL-萨波 100x/0.75 numeric aperture immense objective lens (Leica Microsystems). Images were captured using a SPOT digital camera (Diagnostic Instruments). Biostation IM (Nikon Instruments, Melville, NY) was used to capture live cell images.

**Protein-fragment complementation assay (RePCA)**

Expression vectors pcDNA3.1/Zeo(+) and pcDNA3.1/Hygro(+) were purchased from Invitrogen (Carlsbad, CA). Plasmids PS1941 encoding AKT1-GFP and PS0951 encoding PDK1-GFP were from Bioimage (Soeborg, Denmark). Venus cDNA was kindly
provided by Dr. Miyawaki\(^2\). Venus N terminal fragment (VN) was PCR amplified and cloned into pcDNA3.1/Zeo(+) to make BAIT-VNN-Zeo to express VN (amino acids 1 to 158). AKT1 was amplified from PS1941 and cloned into BAIT-VNN-Zeo to make the construct BAIT-VNN-AKT1-Zeo for expressing VN-AKT1 fusion. Venus C terminal fragment (VC) was PCR amplified and cloned into pcDNA3.1/Hygro(+) to make PREY-VC-Hygro to express VC (amino acids 159 to 239). PDK1 gene was PCR amplified from PS0951 and cloned into PREY-VC-Hygro to make the construct PREY-VC-Hygro-PDK1 for expressing VC-PDK1 fusion. Full-length Vimentin were cloned into PREY-VC-Hygro to express VC-Vimentin fusion protein. All constructs were confirmed by sequencing. Expression of the fusion proteins was assessed by western blots. SKLMS1 cells were transfected to express VN-AKT1 with or without a VC-vimentin fusion protein. Fluorescence was examined by fluorescent microscopy and flow cytometry.

**Immunohistochemistry**

H+E and anti-vimentin immunohistochemical staining of selected human STS specimens were conducted through the MDACC IHC clinical Core.

**Migration and Invasion Assays**

BioCoat cell culture inserts and polycarbonate filters with 8-\(\mu\)m pores (Becton Dickinson Labware) in 24-well tissue culture plates were used for migration assays. Lower chamber compartments contained DMEM supplemented by 1% bovine serum albumin or 1% fetal bovine serum as chemoattractants. Cells (5\(\times\)10\(^4\)) were seeded in the upper compartment and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\) for varying times, as indicated per specific experiment (6-16 h). Invasion assays were

\(^2\) Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, & Miyawaki A (2002) *Nat Biotechnol* 20, 87-90.
conducted similarly using 24-well BioCoat Matrigel invasion chambers with 8-μm pore size polycarbonate filters coated with Matrigel (Becton Dickinson Labware). After incubation, filters were fixed with 4% formaldehyde and stained with 0.2% crystal violet (Baxter Healthcare). Cells on the upper surface of the filters were removed by wiping with a cotton swab, and migratory and invasive activities were determined by counting the number of cells per high-power field (×200) that had migrated to the lower side of the filter.

**Western Blotting and Coimmunoprecipitation**

Briefly, 25-50 μg of proteins extracted from cultured cells or tumor tissues were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked and blotted with relevant antibodies. Horseradish peroxidase–conjugated secondary antibodies were detected by ECL (Amersham Biosciences). IRdye680- and IRdye800-conjugated secondary antibodies (Molecular Probes) were detected using Odyssey Imaging (LICOR Biosciences).

For immunoprecipitation, protein lysates (500-1000 μg) prepared from cultured cells and tumor specimens were used. Immunocomplex pull-down was achieved via overnight incubation of protein lysates with relevant antibodies bound to protein G Sepharose beads (GE Healthcare) at 4°C. After careful washing, loading buffer (Bio-Rad) was added, and the samples were boiled at 100°C for 6 min. Coimmunoprecipitated proteins were then subjected to WB as described above.

**2D PAGE Western Blot**

SKLMS1 cells treated with A563 or DMSO (control) were lysed side-by-side in a commercially available cytosolic extraction buffer (EMD Biosciences) supplemented with a protease inhibitor cocktail. Clear lysates were subsequently cleansed by precipitation
and reconstituted in 200 μl of rehydration buffer (9 M urea, 1 M thiourea, 2% CHAPS, 1% dithiothreitol and 0.2% ampholytes). Protein concentration was determined by the Bradford assay. For the isoelectric focusing (IEF), approximately 200 μg of proteins were applied onto 11-cm IPG strips (pH 3-10), and focusing was performed for a total of 50,000 Vh using the IPGphore IEF system (GE Healthcare). Focused strips were then prepared for conventional SDS-PAGE by sequential coating with SDS for 15 min in reducing buffer (50 mM Tris (pH 6.8), 6 M urea, 2% DTT, 2% SDS, 20% glycerol), followed by 15 min of incubation in alkylating buffer (50 mM Tris (pH 8.8), 6 M urea, 2.5% iodoacetamide, 2% SDS, 20% glycerol). Resolved proteins on SDS-PAGE were transferred onto PVDF membrane by standard procedures and blocked overnight with 10% milk in Tris-buffered saline containing 0.1% Tween 20 (TBST). After three washes with TBST, membrane was incubated with primary antibody (anti-vimentin mouse monoclonal IgG1, 1: 500; Santa Cruz Biotechnology) overnight in 5% bovine serum albumin. These were followed with five washes in TBST and 1 h incubation with secondary antibody (mouse IgG-horseradish peroxidase conjugate). Protein signals were detected with ECL western blotting detection reagents (GE Healthcare).

**In-Gel Trypsin Digestion**

AKT1 and interacting proteins that were coimmunoprecipitated as described above were solubilized in SDS solution (0.3% SDS and 1% mercaptoethanol) and run on SDS-PAGE. After migration, the gels were fixed, and proteins were detected with Coomassie blue staining. Selected Coomassie-stained AKT1 immunoprecipitated protein bands were cut out of the gels and rinsed three times in deionized water. Gel slices (≤1 mm) were incubated with 50 mM ammonium bicarbonate buffer (pH 8.0) for 30 min at 37°C. Following incubation, buffer was replaced with deionized water and further incubated for 30 min. The water was then removed, and samples were then mixed with acetonitrile for
5 min to dehydrate the gel pieces. This procedure was repeated twice, and excess acetonitrile was removed completely in a speed vacuum for 45 min. Finally, dried gels were covered with 10 μl of trypsin solution (10 mg/ml in 25 mM ammonium bicarbonate, pH 8.0), and in-gel digestion was performed at 37°C for 6 h. The digested sample was prepared for mass spectrometry analysis as follows. One microliter of the tryptic digests were spotted directly onto the target plate and allowed to dry. A stock matrix solution (5 mg/ml alpha-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in 50% (v/v) acetonitrile) was diluted two-fold (1:1) with 50% acetonitrile, and 1 μl of the working matrix was then applied on the sample spot and allowed to dry. The dried target spot was blown with compressed air (Decon Labs) before been inserted into the mass spectrometer.

For phosphoproteomics, purified vimentin after incubation with active AKT or without AKT in the presence of cold ATP was separated on SDS-PAGE (in vitro kinase assay described below). Bands were identified via Coomassie blue staining and were isolated, excised, and subjected to in gel-trypsin digestion as described above prior to mass spectrometric analysis.

MALDI-TOF MS was used for protein identification. Data were acquired with an Applied Biosystems 4700 MALDI TOF/TOF Proteomics Analyzer. The instrument was operated in positive-ion reflectron mode, mass range was 850-3,000 Da, and focus mass was set at 1,500 Da. For the MALDI MS data acquisition, 2,000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using known autolytic fragments of trypsin as internal standards. Following MALDI MS analysis, MALDI MS/MS was performed on several (5-8) abundant ions from each sample spot. A 1-kV positive-ion MS/MS method was used to acquire data under post-source decay conditions. The instrument precursor selection window was ±3 Da, and 4,000 laser shots were acquired and averaged for each sample spot.
Applied Biosystems GPS Explorer (v. 3.0) software was used in conjunction with the MASCOT (Matrix Science) search engine to search the eukaryotes taxonomy in the National Center for Biotechnology Information database using both MS and MS/MS spectral data. MS peak filtering included the following parameters: mass range, 800 to 4,000 Da; minimum S/N filter, 10; mass exclusion list tolerance, 0.5 Da. Other search parameters were as follows: maximum missed cleavages were set to unity; fixed modifications included cysteine carbamidomethylation; and variable modification included methionine oxidation, N-terminal acetylation, and pyroglutamylation of N-terminal glutamine and glutamate residues. Precursor tolerance was set at 0.2 Da, MS/MS fragment tolerance was set at 0.3 Da, and peptide mass values were monoisotopic. The significance of a protein match, based on both the peptide mass fingerprint in the first MS and the MS/MS data from several precursor ions, is based on expectation values. The expectation value is the number of matches with equal or better scores that are expected to occur by chance alone. The default significance threshold is p<0.05, so an expectation value of 0.05 is considered to be on this threshold. We used a more stringent threshold of $10^{-3}$ for protein identification; the lower the expectation value, the more significant the score.

**Mass Spectrometry for Phosphorylation Site Analysis**

Three complementary MS-based methods were used for phosphoproteomics: MALDI TOF/TOF, liquid chromatography (LC)/MS/MS, and multiple reaction monitoring (MRM). Tryptic digests of purified vimentin (after incubation with active AKT or without AKT in the presence of cold ATP) were initially run on MALDI TOF/TOF (described above) to generate comparative peptide fingerprinting of the protein and to localize possible post-translational modifications. Aliquots of the digest were further subjected to LC/MS/MS. Reverse-phase capillary chromatography was performed on a Dionex Ultimate LC
system. The sample digests were loaded using a Dionex Famus sample loader, and the peptide components were separated on a PepMap100 C18 75um capillary column. The mobile-phase compositions were 0.1% (v/v) acetic acid for the aqueous phase and 80% (v/v) for the organic phase. The organic phase was linearly increased from 5% to 40% organic over 45 min, with a flow rate to the column of 200 μl/min. The mass spectra were obtained on an Applied Biosystems QTRAP 4000 electrospray mass spectrometer fitted with a nanospray ionization source maintained at 2.5 kV in positive-ion mode. Data were collected in an IDA fashion, with the three most intense doubly to triply charged signals selected to record a tandem mass spectrum of each parent ion for 1 s each in the m/z range of 225-1,500. The spectra were analyzed automatically using the MASCOT search engine with variable phospho (S and T) modifications. MRM experiments were performed to detect potential phosphopeptides using an ABI QTRAP 4000 mass spectrometer. Four MRM transitions were used in this experiment: Q1=788.4 and Q3=739.4 corresponding to the +2 ion of the predicted phosphopeptide TYSLGSALRPSTSR; Q1=526.0 and Q3=493.3 to the +3 ion of this same peptide; Q1=757.3 and Q3=708.3 to the predicted phosphopeptide QVQSLTCEVDALK, Q1=497.8 and Q3=448.8 corresponding to the +2 ion of the predicted phosphopeptide.

**Animal Experiments**

All animal procedures and care was approved by the Institutional Animal Care and Usage Committee of UTMDACC. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." SKLMS1 cells transfected to express either Vim S39A-GFP or Vim S39D-GFP were selected by neomycin, pooled, and FACS sorted by GFP prior to expansion and injection. 1 x 10^6/0.1ml HBSS/mouse were injected subcutaneously (SC) to 6 week-old female SCID mice (NCI/NIH; n=5/per cell line). SC tumors were measured twice weekly by digital
caliper. Study was terminated after 6 weeks, mice were sacrificed, and tumors were measured (tumor volume was calculated as \( V = L \times W^2 \times \pi/6 \), where \( V \) = volume, \( L \) = length, and \( W \) = width).