Figure S1

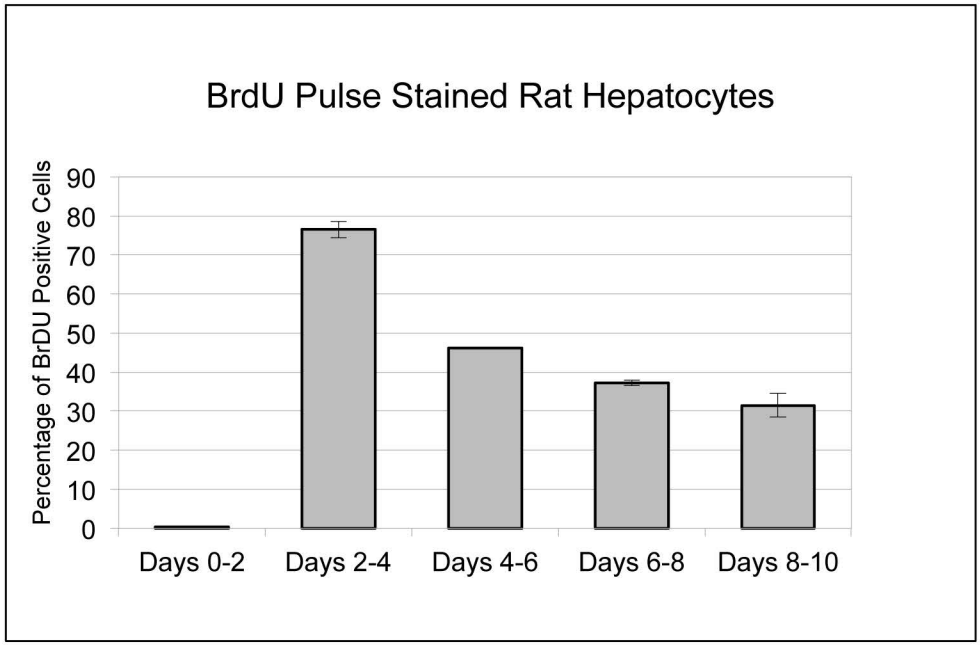

BrdU Pulse Stained Rat Hepatocytes
Percentage of BrDU Positive Cells
90
80
70
60
50
40
30
20
10
0
Days 0-2
Days 2-4
Days 4-6
Days 6-8
Days 8-10

Figure S2

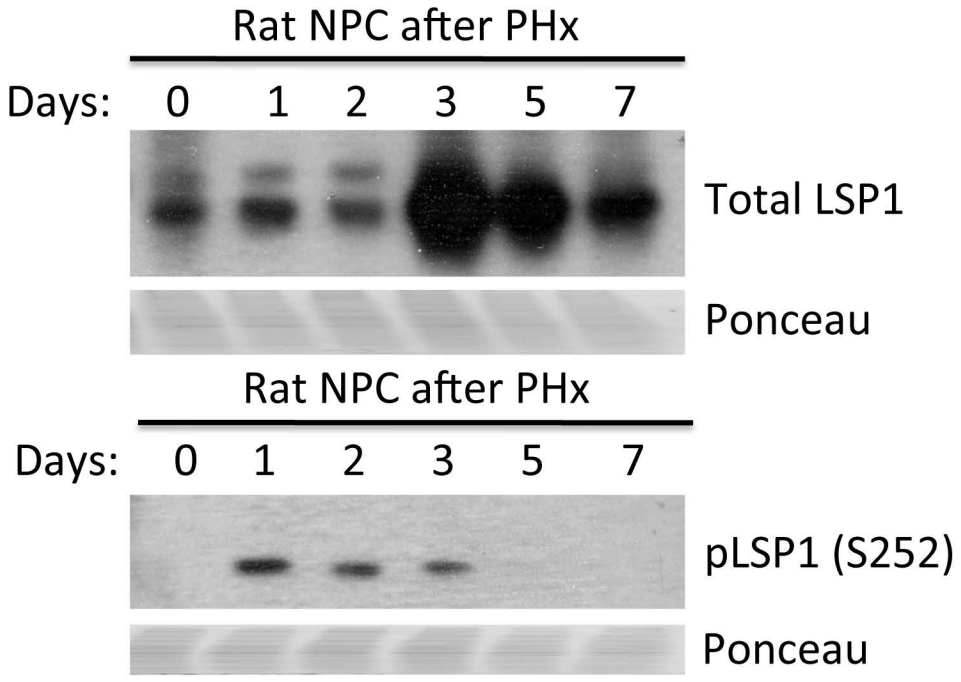

Rat NPC after PHx
Days: 0 1 2 3 5 7
Total LSP1
Ponceau
Rat NPC after PHx
Days: 0 1 2 3 5 7
pLSP1 (S252)
Ponceau

Figure S3

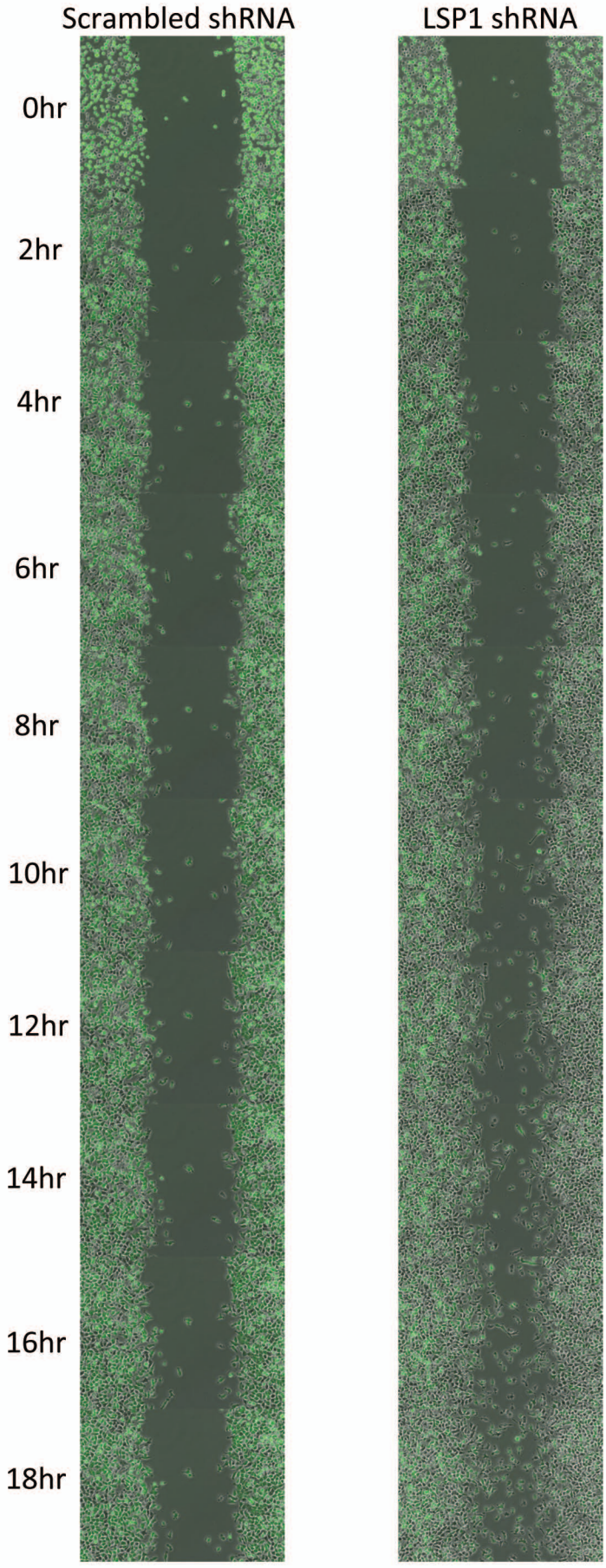
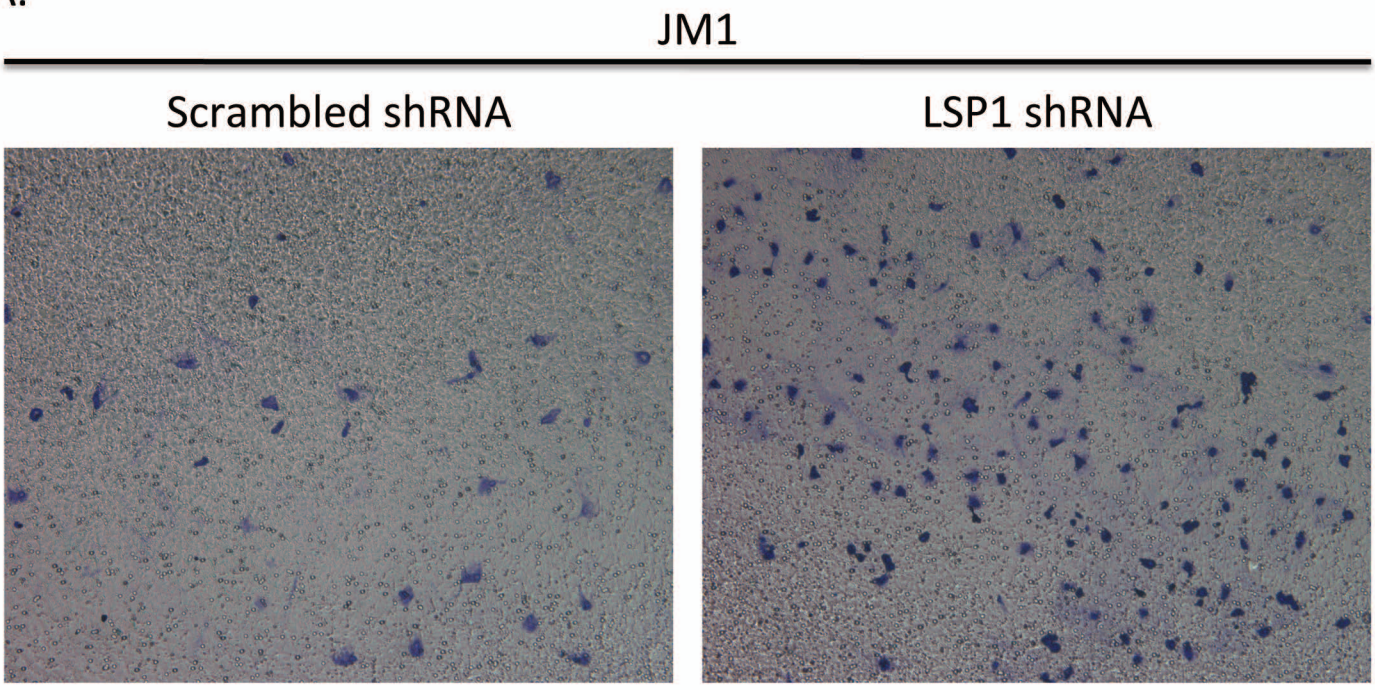

Scrambled shRNA
LSP1 shRNA
0hr
2hr
4hr
6hr
8hr
10hr
12hr
14hr
16hr
18hr

Figure S4

A.


JM1
Scrambled shRNA
LSP1 shRNA

B.

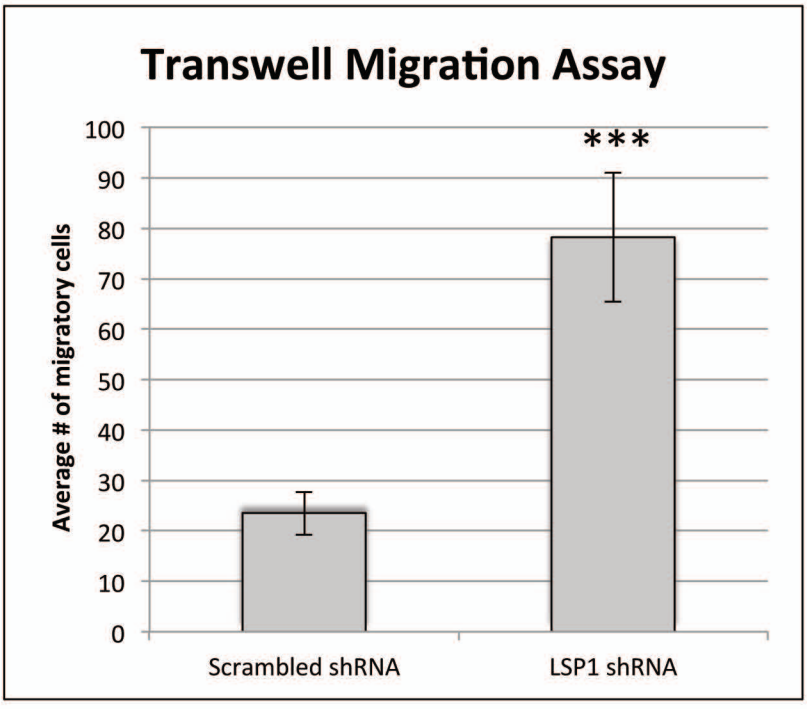
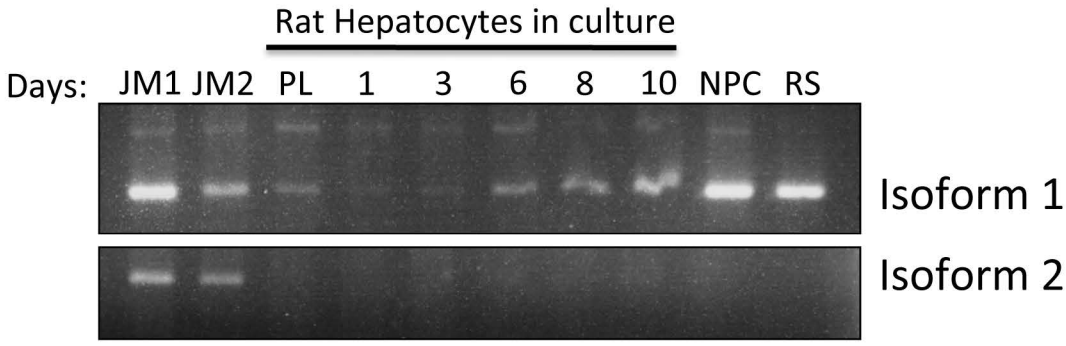

Transwell Migration Assay
Average # of migratory cells
100
90
80
70
60
50
40
30
20
10
0
***
Scrambled shRNA
LSP1 shRNA

Figure S5


Rat Hepatocytes in culture
Days:
JM1
JM2
PL
1
3
6
8
10
NPC
RS
Isoform 1
Isoform 2

Supplemental Materials for Leukocyte Specific Protein-1: a novel regulator of hepatocellular proliferation and migration deleted in human HCC

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## Materials and Methods:

### Cell lines and reagents:

JM1 and JM2 cell lines (1) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Atlas) and gentamicin (1:1000) and maintained in an incubator at 37°C with 5% $CO_2$. Rabbit anti-LSP1 primary antibody was a generous gift from Dr. Jan Jongstra (University Health Network, Toronto, CA). Additional antibodies used in this publication were: cyclin D1(Neomarkers), phospho-LSP1 (S252) (Abcam), KSR (Santa-Cruz), phophoERK1/2 (tyr202/204) (cell signaling technologies), and total ERK1/2 (cell signaling technologies), HNF4α (Santa Cruz) and β-actin (Sigma). GFP tagged rat LSP1 shRNA plasmid and control scrambled shRNA GFP plasmid were purchased from Origene (#TG702934). Rat LSP1 cDNA plasmid was purchased from Open Biosystems Dharmacon (#MRN1768-202784006) GenJet In Vitro DNA Transfection Reagent (Ver. II) was purchased from SignaGen Laboratories.

### Isolation and culture of primary rat hepatocytes

Rat hepatocytes were isolated from normal male Fisher 344 rats using an adaptation of Seglen's calcium two-step collagenase perfusion technique described

previously(2, 3). Isolated rat hepatocytes (300,000 cells/ml) were cultured on collagen-coated six well plates in hepatocyte growth medium supplemented with HGF (40ng/ml) and EGF (20ng/ml)(4, 5). Hepatocyte and NPC pellet were harvested along with hepatocytes cultured for 1, 3, 6, 8 and 10 days for protein and RNA analysis.

**2/3 partial hepatectomy (PHx) of rat liver and isolation of rat hepatocytes after 2/3 PHx**

Male Fisher 344 rats were subjected to 2/3 partial hepatectomy as previously described(4, 6). Rats (n=3 for each time point) were sacrificed on days 1, 2, 3, 5, 7 and 10. Regenerating liver tissue was harvested at the various time points and either snap frozen for protein and RNA analysis, fixed in 10% formalin for paraffin embedding, and embedded into OCT media and frozen at -80°C for cryosectioning. At various time points after PHx, livers were perfused using an adaptation of Seglen's calcium two-step collagenase perfusion techniques as previously described(2, 3). Hepatocyte and NPC pellets were collected for protein and RNA analysis.

**Transfection of JM1 cell line and creation of stable cell line**

JM1 cells were transfected with either GFP-LSP1 shRNA plasmid (Origene, #TG702934) or scrambled shRNA plasmid (Origene) for a control at ~85% confluency in 6 well plates using lipofectamine 2000 (Invitrogen). Briefly, following the manufacturer's protocol, the cells were transfected with 4μg of plasmid DNA in

serum free DMEM overnight. Serum free media was replaced with DMEM containing 10% FBS and 72 hours post transfection, the cells were trypsinized and plated in a 10mm dish and treated with 10µg/ml puromycin (Sigma) for the selection of stably transfected cells. Stable clones were isolated and maintained in the presence of 10µg/ml puromycin.

**Migration Scratch Assay, Transwell Assay and MTT Assay**

Using the JM1 LSP1 shRNA stable cell line, a scratch assay, transwell assay and MTT assay were performed in comparison with native (control) JM1 cells. For the scratch assay, cells were plated at $2x10^5$ cells/well in a 6 well plate and cultured in DMEM + 10% FBS until confluent. Upon confluency, a scratch was made in the monolayer using a 10µl pipette tip and the cells were washed in DMEM to remove unattached cells. An image was taken using an inverted fluorescence microscope at the time of the scratch (time 0) and 24 hours post scratch. For the transwell assay, cells were seeded at $1x10^5$ cells per transwell insert in serum free medium. The bottom chamber of the well contained DMEM with 10% serum. Cells were allowed to migrate for 24 hours at 37°C. After 24 hours, the cells that migrated to the bottom of the transwell membrane were fixed in 4% paraformaldehyde and stained with a 0.1% Coomassie blue/ 10% methanol/ 10% acetic acid solution. Cells that did not migrate through the membrane were carefully removed using a cotton tipped applicator. The membranes were imaged using a Olympus Provis inverted microscope and the cells of at least three fields per membrane were counted using Image J software. For the MTT assay, cells were plated at $1x10^5$ cells/well in 2- 6

well plates and cultured in DMEM + 10% serum. At approximately 50% confluency, the media in both plates was changed to serum free media. Baseline MTT values were measured at 24 hours after addition of serum free medium. Proliferation was stimulated with 10% FBS in DMEM and absorbance measured after 24 h. MTT absorbance after 24 h was normalized to the baseline measurements.

**Transient Transfection of JM2 cells and BrdU incorporation assay**.

JM2 cells were transfected with either pExpress-1 vector alone (control) or rat LSP1 cDNA (Dharmacon #MRN1768-202784006) using GenJet In Vitro DNA Transfection Reagent (Ver. II) (SignaGen Laboratories) at 80% confluency in 6 well plates. The cells were transfected with 4µg of plasmid DNA complexed with 8ul of transfection reagent in DMEM supplemented with 10% FBS. Cells were incubated with transfection complexes for 8 hours and then the medium was changed and 2µl of BrdU (Invitrogen) solution was added to fresh complete medium. At 24 hours post transfection, the cells were fixed in 10% formalin and stained using an antibody against BrdU (Invitrogen) using standard immunohistochemical techniques. Images were taken of the stained cells using Provis Fluoview Microscope and quantified using Image J software.

**Hydrodynamic Tail Vein Injection of Plasmid DNA and 2/3 partial hepatectomy.**

Male FVB mice (4 month old) were subjected to a hydrodynamic tail vein injection of either pExpress-1 control plasmid (n=5) or rat LSP1 cDNA plasmid (n=3). Briefly, 20 µg of enodtoxin free plasmid DNA was diluted in 2ml of 0.9% sterile endotoxin free saline solution. The DNA/ saline solution was injected through the tail vein in 7

seconds. Following a 3 hour recovery period, the mice were subjected to a 2/3 partial hepatectomy, as previously described(7). The livers were harvested 42 hours post hepatectomy and the tissue was processed for paraffin embedding, frozen OCT embedding and protein isolation. All procedures performed on mice and rats were approved under IACUC protocols and conducted in accordance with the National Institute of Health animal care and use guidelines.

**Immunofluorescence**

Frozen rat and mouse liver tissue after PHx were cut into 5-micron thick sections and fixed to glass slides. Tissue was fixed in 5% paraformaldehyde for 5 minutes and washed with PBS. Sections were blocked in 2% bovine serum albumin (BSA) for 45 minutes, washed in 0.5% BSA and incubated in primary antibody (αLSP1 1:50) in 0.5% BSA for 1 hour. Following primary antibody incubation, the tissue was washed in BSA and Cy3 conjugated secondary antibody (1:1000) along with FITC-phalloidin (1:500) was added to the sections for 1 hour. Hoechst dye was used to stain the nuclei and gelvatol was used to fix the glass coverslips to the tissue. Slides were stored at 4°C and images were at the Center for Biologic Imaging at the University of Pittsburgh using an Olympus Fluoview II inverted confocal microscope at both high and low power oil immersion objectives for the rat liver tissue. The mouse liver tissue was co-stained with LSP1 (1:50) and HNF4α (1:50 (Santa Cruz)) and imaged using Olympus Provis inverted epi-fluorescence microscope.

**Immunohistochemistry**

Paraffin embedded liver tissue was sectioned into 5μm sections and stained with Ki67 proliferation marker (ThermoFisher) using the avidin-biotin-peroxidase complex technique (Vectastain ABC kit and DAB peroxidase substrate kit, Vector Laboratories). The sections were counterstained with hematoxylin and eosin (H&E). The stained tissue sections were imaged using Olympus Provis inverted microscope at 200x magnification. The percentage of Ki67 positive hepatocytes was quantified using ImageJ software in at least 10 random fields per tissue section.

**Protein Isolation and Immunoblotting**

Whole cell protein lysates of liver tissue and cells was prepared using 1% sodium dodecyl sulfate (SDS) in RIPA buffer (10mM $Na_2HPO_4$, 10 mM $NaH_2PO_4$, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail (P8340, Sigma), phosphatase inhibitor cocktail I and II (P2850 and P5726, Sigma), 0.26 mg/ml amiloride and 0.05 mg/ml AEBSF) and homogenized. Protein concentrations were determined using Bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL) and 30μg protein was loaded and separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Following transfer, membranes were stained with Ponceau S to evaluate efficient loading and transfer of proteins. Blots were probed with primary and secondary antibodies in Tris-buffered saline (TBS) with Tween 20 containing 5% fish gelatin (Sigma, St. Louis, Mo). The membranes were processed with SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL) and exposed to X-ray film (Lab Product Sales, Rochester, NY).

**Reverse transcriptase PCR (RT-PCR)**

RNA was obtained by homogenizing rat livers, hepatocytes and cell lines in Trizol ® reagent (Invitrogen). RNA was DNase treated using the DNase free Kit from Ambion in order to remove any contaminating genomic DNA. Two micrograms of total DNase treated RNA from each sample was reverse transcribed into cDNA using Superscript reverse transcriptase (Invitrogen). PCR using primers specific for LSP1 and GAPDH was performed using Taq polymerase (Invitrogen) on a Thermo Hybaid PCR sprint thermal cycler (Thermo Scientific).

**Immunoprecipitation**

Five hundred micrograms of whole cell lysates from JM1 cells was prepared in RIPA buffer and diluted to a final volume of 500µl. Protein complexes were immunoprecipitated with 10µg of LSP1 (Santa Cruz), F-actin (Abcam), and KSR (Santa Cruz) antibodies overnight at 4°C with end-over-end mixing followed by incubation with protein A/G beads (Santa Cruz) overnight at 4°C. Complexes were centrifuged at 1,000xg for 5 minutes and washed three times in RIPA buffer before resuspension in 2x loading buffer. Immunoprecipitated protein complexes were separated by SDS-PAGE electrophoresis and transferred to Immobilon-P membranes (Millipore). Blots will be probed with antibodies for total LSP1 using rabbit polyclonal serum (generous gift from Dr. Jan Jongstra) kinase suppressor of Ras (KSR) (Santa Cruz Biotechnology) and f-actin (Abcam).

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