AMPK-Independent LKB1 Activity Is Required for Efficient Epithelial Ovarian Cancer Metastasis

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**ABSTRACT**

Epithelial ovarian cancer (EOC) spreads by direct dissemination of malignant cells and multicellular clusters, known as spheroids, into the peritoneum followed by implantation and growth on abdominal surfaces. Using a spheroid model system of EOC metastasis, we discovered that Liver kinase B1 (LKB1), encoded by the **STK11** gene, and its canonical substrate AMP-activated protein kinase (AMPK) are activated in EOC spheroids, yet only LKB1 is required for cell survival. We have now generated **STK11**-knockout cell lines using normal human FT190 cells and three EOC cell lines, OVCAR8, HeyA8, and iOvCa147. **STK11**KO did not affect growth and viability in adherent culture, but it decreased anchorage-independent growth of EOC cells. EOC spheroids lacking LKB1 had markedly impaired growth and viability, whereas there was no difference in normal FT190 spheroids. To test whether LKB1 loss affects EOC metastasis, we performed intraperitoneal injections of OVCAR8-, HeyA8-, and iOvCa147-**STK11**KO cells, and respective controls. LKB1 loss exhibited a dramatic reduction on tumor burden and metastatic potential; in particular, OVCAR8-**STK11**KO tumors had evidence of extensive necrosis, apoptosis, and hypoxia. Interestingly, LKB1 loss did not affect AMPK or phosphorylation in EOC spheroids and tumor xenografts, indicating that LKB1 signaling to support EOC cell survival in spheroids and metastatic tumor growth occurs via other downstream mediators. We identified the dual-specificity phosphatase DUSP4 as a commonly upregulated protein due to LKB1 loss; indeed, DUSP4 knockdown in HeyA8-**STK11**KO cells partially restored spheroid formation and viability.

**Implications:** LKB1 possesses key tumor-promoting activity independent of downstream AMPK signaling during EOC metastasis.

**Introduction**

Epithelial ovarian cancer (EOC) is a highly lethal malignancy in women since nearly all patients are diagnosed with metastatic disease, at which point the 5-year survival rate is only 30% (1). Standard-of-care for late-stage EOC has remained largely unchanged over 25 years and encompasses aggressive cytoreductive surgery with combination carboplatin/paclitaxel chemotherapy. Most patients initially respond to treatment, but recurrence and chemoresistance is common and fatal in most cases. Therefore, the development of novel approaches to impede metastasis and recurrence of chemoresistant disease will be paramount to improve outcomes for women diagnosed with EOC (2).

A common symptom of advanced EOC is the accumulation of ascites within the peritoneal cavity (3). This ascites often harbors individual malignant cells or those existing as multicellular aggregates, herein referred to as spheroids, the latter of which offer numerous selective advantages in the context of metastatic dissemination (4). EOC spheroids exhibit anoikis resistance and possess an enhanced capacity to adhere to ECM and displace mesothelial cell monolayers (3, 5, 6). In addition, recent evidence indicates that EOC spheroids seed peritoneal metastases that maintain the cellular heterogeneity of the primary tumor (7). Thus, EOC spheroids likely play a key role in efficient intraperitoneal metastasis.

We previously reported that Liver kinase B1 (LKB1, encoded by the **STK11** gene) is expressed in the majority of EOC cell lines and patient ascites-derived cells; importantly, transient knockdown of **STK11** in EOC cells results in reduced spheroid viability and increased chemosensitivity (8). LKB1 is a ubiquitously expressed, highly conserved serine-threonine kinase that commonly acts as the upstream kinase for AMP-activated protein kinase (AMPK), primarily in regulating cell metabolism (9). The LKB1–AMPK signaling axis functions as a sensor of energy status in the cell and enables adaptation to conditions that deplete intracellular ATP. Decreased ATP and the corresponding increase in AMP favors phosphorylation and activation of AMPK by LKB1, resulting in the coordinated downregulation of anabolic pathways and upregulation of catabolic pathways to restore energy homeostasis (10).

LKB1 is often described as having tumor suppressor-like activity because specific cancers feature somatic alterations in the LKB1-encoding gene **STK11** (11–13) or have decreased LKB1 protein expression (14, 15). In addition, germline-inactivating mutations in **STK11** have been implicated in Peutz-Jeghers syndrome (16), which is characterized by the formation of benign polyps in the gastrointestinal tract and an increased risk for several cancers (17). LKB1 loss has also been implicated in EOC initiation (15, 18); however, there is significant evidence suggesting that LKB1 and its direct signaling partners function in context-dependent, protumorigenic capacities (8, 19–22).

To date, no studies have assessed the impact of LKB1 loss-of-function in mouse models of late-stage EOC. In this study, we sought to determine how complete LKB1 ablation in EOC cells would affect metastatic potential. We present evidence indicating that LKB1 loss...
decreases anchorage-independent viability of EOC cells and reduces the long-term spheroid maintenance. In a similar fashion, LKB1 loss in three independent EOC cell lines significantly reduced tumor burden and extended host survival in a xenograft mouse model of peritoneal metastasis. On the basis of these findings, we propose that strategies targeting LKB1 or its signaling partners would have therapeutic potential in metastatic EOC.

Materials and Methods
Cultured cell lines
OVCAR8 and HeyA8 cell lines were cultured in RPMI1640 (Wisent). FT190 and iOvCa147 cell lines were cultured in DMEM/F12 (Life Technologies). For all cell lines, growth medium was supplemented with 10% FBS. OVCAR8 and HeyA8 cells were obtained from the ATCC; iOvCa147 cells were generated by us as described previously (23). The immortalized human fallopian tube secretory epithelial cell line FT190 (24) was provided by R. Drapkin (University of Pennsylvania, Philadelphia, PA). All cell lines were authenticated by short tandem repeat analysis performed by The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, Ontario, Canada), and routinely tested for Mycoplasma using the Universal Mycoplasma Detection Kit (30-1012K, ATCC) or as performed by IDEXX BioResearch prior to xenograft experiments.

Generation of STK11 KO cell lines
The 20-nucleotide guide sequence targeting the STK11 gene 5’-AGCTT GGCCC GCTTG CGGCG-3’ was selected using CRISPR Design Tool (https://tools.genome-engineering.org). Complementary oligonucleotides 5’-CACCG AGCTT GCCGG GCCGGC-3’ and 5’-AAACCC GCGGCAG GGCGGAGCTC-3’ (Sigma-Genosys) were annealed and ligated into the BbsI-digested restriction endonuclease site of pSpCas9(2B)-2A-Puro plasmid (gift from Dr. F. Dick, Western University, London, Ontario, Canada) as per the protocol described in Ran and colleagues (25) to generate the pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid.Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid. Cells were transfected with pSpCas9-sgSTK11 plasmid.

Generation of DUSP4-overexpressing cell lines
FT190, OVCAR8, HeyA8, and iOvCa147 cells were transfected with a plasmid to drive expression of a human DUSP4-Myc-DDK fusion protein (Origene; catalog no. RC200640) or empty vector. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, followed by treatment with 1 μg/mL puromycin for one day. After growth recovery, limiting dilution cloning of potential STK11-knockout cells was performed and confirmation of STK11 knockout by Western blotting for LKB1 expression. A minimum of five clones lacking LKB1 protein expression were positively identified and subsequently mixed in equal ratios to generate the STK11KO cell line populations.

Antibodies and reagents
Antibodies against LKB1 (#30505), phospho-JNK (T183/Y185; #4668P), phospho-p38 (T180/Y182; #4511S), and DUSP4 (#5149S) were purchased from Cell Signaling Technology and used at 1:3,000 in 5% BSA/TBS-T. Antibody against CA9 (AF2188; 1 μg/mL) was purchased from R&D Systems. Antibody against tubulin (T5168; 1:8,000) and vinculin (V9264; 1:50,000) were purchased from Sigma. HRP-conjugated antibodies against mouse IgG (NA931V; 1:10,000) and rabbit IgG (NA934V; 1:10,000) were purchased from Sigma. IHC was performed by Molecular Pathology (Robarts Research Institute, London, Ontario, Canada). Antibody against Ki67 (ab1667; 1:200) was purchased from Abcam. Antibody against cleaved caspase-3 (#9661; 1:50) was purchased from Cell Signaling Technology and used according to Molecular Pathology standard protocols. Oligomycin A (#11342) and AICAR (#10010241) were purchased from Cayman Chemical; STO-609 (#73862) was purchased from Stemcell Technologies.

Immunoblot analysis
Isolation of protein lysates and Western blotting were performed as described previously (8).

Doubling time determination in adherent culture
Cells were seeded into 96-well adherent culture plates at a density of 1,000 cells per well. Phase-contrast images were captured at 3-hour intervals for a total of 96 hours and confluence was measured using the IncuCyte Zoom imaging platform. Doubling time was determined by fitting an exponential growth curve to the confluence-over-time data (GraphPad Prism 6.05).

Clonogenic assays
Cells were seeded at specific cell densities then fixed and stained when cells formed well-defined colonies with minimal overlap. OVCAR8 and OVCAR8-STK11KO cells: 250 cells/35-mm well, 8 days; iOvCa147 and iOvCa147-STK11KO cells: 1,000 cells/35-mm well, 8 days; HeyA8 and HeyA8-STK11KO cells: 2,500 cells/10-cm plate, 14 days; FT190 and FT190-STK11KO cells: 1,000 cells/10-cm plate, 12 days. Cells were fixed and stained using the Protocol Hema 3 staining kit (Thermo Fisher Scientific). Colony number and size were determined using the Fiji Is Just ImageJ (Fiji) image processing software package (26); the Trainable Weka Segmentation plugin (27) was used to generate classifiers to segment images into stained colonies and background. Colony number and area were determined using the “analyze particles” function to outline each colony as a region of interest and provide colony counts.

Soft agar assays
EOC cells were suspended at a density of 25,000 cells/1.5 mL in medium supplemented with 10% FBS and 1% melted soft agar; this suspension was added to each well containing solidified 2% agar. Additional growth medium was then added to each well, and plates were incubated for 14 days. Images of random fields of view were captured at 25× magnification using a Leica DMI 4000B inverted microscope. Colony number and size were determined using the Fiji image processing software package as described above.

Spheroid viability assay
Cells were seeded into 24-well ULA cluster plates (50,000 cells per well in a volume of 1 mL). At the specified time points, spheroids were collected and trypsinized at 37°C with gentle agitation every 10 minutes until aggregates were no longer visible (10–30 minutes). Trypsin was inactivated and Trypan Blue Exclusion cell counting was performed using a TC10 Automated Cell Counter (Bio-Rad). For
long-term spheroid growth assays, cells were seeded into a 96-well round-bottom ULA plate at a density of 2,000 cells/well. Phase-contrast images were captured at 2-hour intervals for up to 12 days using the IncuCyte Zoom imaging platform. Single spheroids were isolated at specific time points postseeding (3, 7, 14, and 21 days) and transferred into separate wells of a 48-well adherent culture plate. Incubated for 2 days, then attached spheroids were fixed and stained using the Protocol Hema 3 staining kit (Thermo Fisher Scientific).

Xenotransplantation assays
NOD/SCID female mice (8–10 weeks old; Charles River Laboratories) were inoculated by intraperitoneal injection with 150 μL of PBS containing the following numbers of cells: OVCA8/OVCAR8-STK11KO, 4 × 10⁶; HeyA8/HeyA8-STK11KO, 1 × 10⁶; iOvCa147/iOvCa147-STK11KO, 2 × 10⁶. For survival analyses (OVCAR8, OVCAR8-STK11KO, HeyA8, HeyA8-STK11KO, iOvCa147, and iOvCa147-STK11KO cells), mice were monitored daily after intraperitoneal injection and euthanized using standard criteria for humane endpoints (i.e., lethargy, hunched posture, impaired breathing, extreme weight loss, excessive ascites). Mice were provided chow and water ad libitum throughout the study. All animal experiments were approved by Institutional Animal Care and Use Committee of the University of Western Ontario (London, Ontario, Canada) and carried out in accordance with the approved guidelines.

Xenograft tumor histology and IHC
Immediatley following euthanization of mice, peritoneal tumors were excised and placed into a Formalde-Fresh solution on ice (Thermo Fisher Scientific); samples were incubated in this solution at 4°C overnight. The following day, tumors were washed twice in cold PBS and stored in 70% ethanol at 4°C. Tumors were then embedded in paraffin and sectioned (5 μm thickness). Hematoxylin/eosin staining was used to visualize tissue architecture, and adjacent sections were immunohistochemically stained to assess Ki67 and cleaved-caspase-3, with hematoxylin as a counterstain. All processing steps after storage in 70% ethanol were performed by the Molecular Pathology Core Facility at Robarts Research Institute (London, Ontario, Canada). Images of stained tumor sections were then captured using an Aperio ScanScope slide scanner (Leica).

IHC analysis and scoring
IHC analysis was performed using the Fiji distribution of ImageJ (26). For quantification of Ki67 staining, xenograft tumor section boundaries were defined as a region of interest, and 6–10 200× power fields of view, all of equal area, were randomly generated. Ki67-positive nuclei were masked using the Trainable Weka Segmentation plugin (27), and masked regions were counted using a minimum power threshold of 0.25. Positive nuclei were masked using the Trainable Weka Segmentation plugin (27), and masked regions were counted using a minimum power threshold of 0.25. Positive nuclei were masked using the Trainable Weka Segmentation plugin (27), and masked regions were counted using a minimum power threshold of 0.25. Positive nuclei were masked using the Trainable Weka Segmentation plugin (27), and masked regions were counted using a minimum power threshold of 0.25. Positive nuclei were masked using the Trainable Weka Segmentation plugin (27), and masked regions were counted using a minimum power threshold of 0.25.

Inhibition of CAMKK2
Cells were seeded into a 6-well adherent culture plate, and treatment was initiated the following day with 10 μmol/L STO-609 or an equivalent volume of DMSO. For analysis of spheroids, cells were seeded into a 6-well ULA plate and treated with 10 μmol/L STO-609 or DMSO at the time of seeding. Lysates were generated for immunoblot analysis at 24 hours (FT190, FT190-STK11KO, iOvCa147, and iOvCa147-STK11KO cells) or 72 hours (OVCAR8 and OVCAR8-STK11KO cells).

Induction of metabolic stress
For glucose deprivation experiments, cells were seeded into 96-well adherent culture plates (2,500 cells/well) in complete growth medium. The following day, growth medium was aspirated, washed once with serum- and glucose-free medium, and replaced with medium containing 10% dialyzed FBS (Invitrogen) and 0.1 g/L glucose. For serum deprivation experiments, cells were incubated with medium containing 0.25% FBS. For inhibition of mitochondrial complex V using oligomycin A or pharmacologic activation of AMPKα using AICAR, cells were seeded into 96-well adherent culture plates (1,000 cells/well) in complete growth medium in a volume of 150 μL. The following day, 50 μL of complete growth medium containing oligomycin, AICAR, or vehicle (DMSO) was added to each well. Viability was assessed 72 hours later using the CyQuant Cell Proliferation assay (Thermo Fisher Scientific) as per manufacturer’s instructions.

Reverse-phase protein array analysis
Adherent cells were harvested at 24 and 72 hours postseeding by trypsinization followed by addition of RPMI with 10% FBS; spheroids were directly collected from ULA plates at these same time points. Cells were pelleted (800 × g, 4°C) and washed twice in ice cold PBS, then flash-frozen on dry ice, and stored at −80°C until sample submission. Reverse-phase protein array (RPPA) analysis was performed by the Functional Proteomics RPPA Core Facility at the MD Anderson Cancer Center according to their standard protocol (https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppa-core/antibody-information-and-protocols.html). Normalized data and log-transformed fold changes are summarized in Supplementary Table S2. A heatmap was generated using log-transformed fold change data and the Matrix2png interface (https://matrix2png.msl.ubc.ca).

DUSP4 knockdown
Transfections were performed of cells seeded in 6-well plates using DharmaFECT1 as per manufacturer’s protocol (Dharmacon; Thermo Fisher Scientific Inc.). DUSP4 siGENOME SMARTpool (M-003963-03), two siGENOME siRNAs targeting the DUSP4 ORF (D-003963-03 and D-003963-05), and nontargeting control pool #2 (D-01206-14-05) siGENOME SMARTpool siRNA were used. After 72 hours, trypsinized cells were counted and seeded at a density of 5 × 10⁴ cells per well in 24-well ULA cluster plates and 2 × 10³ cells per well in 96-well round-bottom ULA cluster plates. Cell viability was determined at 72 hours postseeding in 96-well round-bottom ULA cluster plates using CellTiter-Glo (Promega). Western blot analysis was performed on adherent cell lysates at 72 hours postseeding to confirm DUSP4 knockdown.

Statistical analysis
All statistical analyses were performed using GraphPad Prism 6.05 (GraphPad Software). The results from in vitro analyses were assessed using a two-tailed Student t test or two-way ANOVA with Tukey multiple comparisons test. Tumor latency analysis in mice was tested for significance using the log-rank test. In all cases, a P value of less than 0.05 was considered statistically significant.

Results
LKB1 lacks tumor suppressor-like activity in EOC
We previously reported that LKB1 protein is expressed in EOC tumor samples, and that transient STK11 knockdown decreases EOC spheroid viability and chemoresistance, suggesting that LKB1 or its
signaling partners might represent viable therapeutic targets in EOC (8). Therefore, we sought to investigate how sustained LKB1 loss would affect tumorigenic and metastatic potential in several different EOC cell lines. OVCAR8 (high-grade serous ovarian cancer; HGSOC) and HeyA8 (poorly differentiated ovarian cancer) cells form robust spheroids as well as aggressive tumor growth in immune-compromised mice, and therefore represent two EOC cell lines with which to test our hypothesis regarding LKB1 function. The iOvCa147 cell line was derived by our group from the ascites of a heavily treated HGSOC patient (23). This cell line forms less dense yet viable spheroids, and establishes ascites and tumors in NOD/SCID mice with longer latency as compared with OVCAR8 and HeyA8 cells. We used CRISPR/Cas9-based genome editing to generate stable cell lines lacking LKB1 expression: OVCAR8-\textit{STK11}KO, HeyA8-\textit{STK11}KO, and iOvCa147-\textit{STK11}KO cell lines cultured in soft agar to assess anchorage-independent colony forming potential. Images are representative of three independent experiments. Scale bars, 500 μm. D, Quantification of soft agar colony formation. Data are presented as colony count and colony size per field of view within each independent experiment and the mean. Groups were compared using the two-tailed Student \( t \) test (\( \ast \), \( P < 0.05; \ast \ast \), \( P < 0.01; \ast \ast \ast \), \( P < 0.0001 \)).

It is well-established that the majority of HGSOC likely originate in the secretory epithelium of the distal fimbriae of the fallopian tube (29–31), and mouse models also support this as a primary cell-of-origin in this disease (32, 33). Two reports have implied that loss of LKB1 expression may be involved in the development of HGSOC (15, 18), whereas we have alternative conflicting data demonstrating that LKB1 is in fact expressed and functional in late-stage EOC (8). If LKB1 has tumor-suppressive properties in EOC, then \textit{STK11} knockout should enhance normal FT190 cell clonogenicity in both anchorage-dependent and -independent conditions. We performed clonogenic assays using each line harboring LKB1 loss to determine whether anchorage-dependent replicative potential would be affected by loss of LKB1 in FTE and EOC cells. We found that LKB1 loss significantly decreased clonogenic potential in FT190 cells, but not in any EOC cell lines tested (Supplementary Fig. S2).

Because anchorage independence is a common feature of transformed cells and critical to EOC metastasis, we determined how LKB1 loss would affect anchorage independence in normal FTE and transformed EOC cells. In contrast to the results from adherent clonogenic assays, loss of LKB1 significantly decreased clonogenic potential in FT190 cells, but not in any EOC cell lines tested (Supplementary Fig. S2).
and was thus not tested by this assay. Furthermore, FT190 cells, which have been immortalized due to p53 and pRB inactivation by SV40 large T antigen (24), were unable to establish any colonies in soft agar with or without intact LKB1 (data not shown). These results suggest that LKB1 does not possess overt tumor-suppressive activity but may be required for aggressive EOC cell growth or viability when in suspension.

LKB1 is required in EOC spheroids to maintain cell viability

Spheroids are frequently present in the peritoneal ascites of patients with EOC, and facilitate peritoneal metastasis because they exhibit anoikis resistance, adhere to mesothelial cell monolayers (3, 5), and help to establish peritoneal metastases (7). We have demonstrated previously that decreased LKB1 expression by transient STK11 knockdown has little to no effect on the growth and viability of adherent proliferating EOC cells, yet viability is reduced in spheroids (8). Thus, we sought to determine how sustained loss of LKB1 would affect FTE and EOC spheroid viability. We cultured FTE and EOC cell lines in suspension using ultra-low attachment (ULA) vessels (Fig. 2A). LKB1 loss significantly decreased the number of viable cells for all EOC cell lines tested (Fig. 2B), indicating that LKB1 is required for maximal EOC spheroid viability in this in vitro model of metastasis. Normal FT190 cells exhibited rapid attrition in spheroid culture (Fig. 2A and B) regardless of LKB1 status, indicating again that LKB1 loss does not possess tumor suppressor-like activity when FTE cells are deprived of attachment to a substratum. Long-term spheroid culture of HeyA8 and OVCAR8 cells yielded an overt decrease in growth and viability due to LKB1 loss (Supplementary Videos), and this was especially pronounced when spheroids were maintained for up to
LKB1 is required to maintain EOC metastatic potential

The results of our in vitro studies indicate that LKB1 is required for both anchorage-independence and spheroid viability in EOC cells. Because spheroids play an important role in EOC metastasis, we sought to determine the effect of LKB1 loss in an in vivo xenograft model of peritoneal metastasis. EOC cells are injected directly into the peritoneal space of immunodeficient host mice to mimic metastasis, where tumor pathology and pattern of dissemination are similar to late-stage disease in patients (34). We hypothesized that stable LKB1 ablation in EOC cells would decrease metastatic potential in vivo.

We found that survival was extended in hosts for all three STK1 KO cell lines when compared with parental cell lines (Fig. 3A). Compared with parental control hosts, median survival was 48.5% longer for OVCAR8-STK1 KO hosts (78 days vs. 52 days; log-rank test, P = 0.0005), but only 17.1% longer for HeyA8-STK1 KO hosts, which approached statistical significance (51 days vs. 41 days; log-rank test, P = 0.1105). Median survival for iOvCa147-STK1 KO hosts was undetermined because most mice survived until study completion, yet survival was significantly longer than the parental control group (log-rank test, P = 0.0015). Frequency of ascites accumulation was decreased in OVCAR8-STK1 KO relative to OVCAR8-injected mice, but overall dissemination patterns were similar, indicating that metastatic trajectory was unchanged by LKB1 loss (Fig. 3B; Supplementary Table S1). Macroscopic tumor burden in OVCAR8-STK1 KO–injected hosts was significantly decreased compared with OVCAR8 cells (Fig. 3B). In mice injected with HeyA8-STK1 KO cells and HeyA8 controls, we observed an altered pattern of tumor dissemination associated with...
LKB1 loss. HeyA8–STK11 KO-injected hosts exhibited a thin layer of tumor cells adherent to the peritoneal wall, and little evidence of solid tumor lesions at other sites (Fig. 3B; Supplementary Table S1). In contrast, HeyA8-injected hosts had several large and solitary solid tumor masses at the omentum and lower peritoneal cavity, often adherent to the intestine and uterus (Fig. 3B; Supplementary Table S1). This resulted in a total tumor burden that was significantly reduced in HeyA8–STK11 KO–injected mice as compared with HeyA8 controls (Fig. 3C). Sustained LKB1 loss was confirmed by immunoblotting of protein lysates from excised OVCAR8- and OVCAR8–STK11KO tumors (Fig. 3D).

Similar to our in vitro spheroid assays, we observed the most striking effects of LKB1 loss in the iOvCa147 cell line, which was originally generated from the ascites of a patient with HGSOC by our group (23). In line with the extended survival of iOvCa147–STK11KO hosts (Fig. 3B), peritoneal colonization was substantially diminished, with most mice exhibiting little evidence of macroscopically observable peritoneal tumors upon necropsy (Fig. 3B). In contrast to iOvCa147–STK11KO hosts, mice injected with iOvCa147 parental control cells exhibited peritoneal tumors at multiple sites, with particularly frequent colonization at the omentum, peritoneal wall, and diaphragm. Because most iOvCa147–STK11KO mice failed to develop tumors, we did not measure overall tumor burden using a single time point analysis in this cohort.

Histologic analysis indicated that OVCAR8–STK11KO tumors nodules frequently contained large necrotic regions, and IHC staining revealed that Ki67-positive cells were frequently restricted to the periphery of tumor nodules with a core of cleaved caspase-3–positive apoptotic cells (Fig. 4A). In line with this, OVCAR8–STK11KO tumors exhibited decreased Ki67 positivity relative to controls (Fig. 4B). Compared with parental OVCAR8 controls, these highly necrotic/apoptotic OVCAR8–STK11KO tumors had increased carbonic anhydrase 9 protein expression (Fig. 4C and D), which is indicative of elevated hypoxia in tumors (35). In contrast, enhanced necrosis and
apoptosis was not evident in HeyA8-STK11 KO tumor xenografts (Fig. 4A), and no difference in Ki67 positivity was observed relative to HeyA8 tumors (Fig. 4B). Very few iOvCa147-STK11 KO hosts developed any tumors (Fig. 3A; Supplementary Table S1). However, histologic analysis of a single iOvCa147-STK11 KO lesion revealed thinly-deposited EOC cells with papillary features that were not evident in the solid tumors arising in iOvCa147 controls (Fig. 4A). Subsequent analysis of Ki67 positivity by IHC showed no significant difference between iOvCa147- STK11 KO tumors and parental controls (Fig. 4B). We also performed automated scoring of cleaved caspase-3 IHC staining as a measure of apoptosis. In all cases, no differences were found between STK11 KO tumors and parental controls (Supplementary Table S3). This suggests that compared with necrosis, apoptosis is unlikely to contribute to the decreased metastatic potential relative to controls.

Notably, the significant decrease in in vivo metastatic potential for all three STK11 KO EOC cell lines corresponded very well with our in vitro results using the spheroid model of metastasis (Fig. 2). Furthermore, in cases where ascites was recovered from host mice, we observed the presence of multicellular aggregates that resembled the morphology of spheroids generated in vitro (Supplementary Fig. S3). This indicates that spheroid biology in our in vitro model recapitulates the phenotype of spheroids formed in an established in vivo model of EOC metastasis.

**LKB1-independent AMPK phosphorylation in EOC cells, spheroids, and xenograft tumors**

We demonstrated previously that both LKB1 and phosphorylated AMPKα are coordinately upregulated in EOC spheroids (8), suggesting that LKB1-AMPK signaling is activated during EOC metastasis. Because LKB1 is commonly described as the major upstream kinase responsible for phosphorylation of AMPKα at Thr172 (9), we expected that phospho-AMPKα would be substantially decreased or ablated in STK11 KO spheroids relative to parental controls. Instead, we found that phospho-AMPKα Thr172 was maintained in spheroids for all STK11 KO cell lines tested (Fig. 5A), indicating that in FTE and EOC
spheroids, AMPK activity is phosphorylated by one or more other kinases independently of LKB1.

Ca(2+)/calmodulin-dependent kinase kinase-beta (CAMKK2) is one principal alternative AMPK upstream kinase (36), and cell detachment can rapidly induce phospho-AMPK at T172 in a CAMKK2-dependent manner in breast cancer cells (37). Therefore, we treated FT190-STK11KO, OVCAR8-STK11KO, and iOvCa147-STK11KO spheroids and parental cell lines with STO-609, a potent cell-permeable inhibitor of CAMKK2 (38). STO-609 substantially decreased phospho-AMPK activity for all cell lines tested (Fig. 5B), indicating that CAMKK2 is likely the major activating kinase for AMPK in FTE and EOC cells, although LKB1 is present or import. Importantly, analysis of tumor xenografts revealed that phospho-AMPK was unaffected by loss of LKB1, also (Fig. 5C and D). Indeed, LKB1 loss had no effect on OVCAR8 cells when subjected to several metabolic stressors—glucose deprivation, serum starvation, mitochondrial inhibition, and AMP mimetic treatment—which act through AMPK activity (Supplementary Fig. S4).

Taken together, these results imply that any of our observed phenotypic effects due to LKB1 loss in FTE and EOC cells and spheroids, and during EOC metastasis in mice, are most likely AMPK independent.

**DUSP4 is upregulated due to sustained LKB1 loss**

Because it appeared that AMPK was not mediating the phenotypic changes in EOC spheroids and tumors due to LKB1 loss, we sought to discover other potential effectors. To do so, we performed RPPA analysis comparing STK11KO knockout cells grown in adherent and spheroid culture using both OVCAR8 and HeyA8 cells. From these data, we observed distinct differences in protein expression between the OVCAR8 and HeyA8 spheroids due to LKB1 loss (Fig. 6A; Supplementary Table S2), which may reflect the differences seen in spheroid culture and xenograft experiments between these two distinct EOC cell lines. However, one protein that was consistently increased due to LKB1 loss in both adherent cells and even more so in spheroids was dual-specificity phosphatase 4 (DUSP4). We observed a 1.86- to 2.06-fold increase in OVCAR8-STK11KO adherent cells and a 1.76- to 2.57-fold increase in spheroid culture; in HeyA8-STK11KO cells, DUSP4 protein was elevated by 2.37- to 2.76-fold in adherent and 2.84- to 3.12-fold in spheroids. We confirmed this increase in DUSP4 protein expression among all three EOC cell lines where LKB1 was knocked down (Fig. 6B). In addition, DUSP4 was increased significantly in OVCAR8-STK11KO xenografts as compared with tumors derived from OVCAR8 cells (Fig. 6C and D).

DUSP4 is a dual specificity phosphatase that typically targets several MAPKs to decrease their phosphorylation state and thus downstream signaling activity (39). To this end, we performed immunoblot analysis of phosphorylated ERK, JNK, and p38 among the three EOC cell lines lacking LKB1 compared with controls. Interestingly, phospho-ERK1/2 was consistently decreased in EOC cells upon spheroid culture; however, levels of phospho-p38 and phospho-JNK (p54 and p46) were either unchanged or different among the three cell lines (Supplementary Fig. S5). Levels of phosphorylated ERK, JNK, and p38, however, yielded no change due to LKB1 loss even though DUSP4 protein is universally increased in these cell lines. To further investigate whether upregulation of DUSP4 protein may impact the spheroid phenotype (i.e., reduced formation potential and cell viability) due to LKB1 loss, we performed transient DUSP4 knockdown in HeyA8 and HeyA8-STK11KO cells (Fig. 6E) and subsequently formed spheroids. We observed a partial restoration of intact spheroid formation by DUSP4 knockdown in HeyA8-STK11KO spheroids (Fig. 6F), as well as increased cell viability (Fig. 6G). To complement this loss-of-function result, we overexpressed epitope-tagged DUSP4 in FT190, OVCAR8, HeyA8, and iOvCa147 cells (Supplementary Fig. S6A), but found no effects on spheroid formation (Supplementary Fig. S6B) or viability (Supplementary Fig. S6C). This indicates that elevated DUSP4 on its own is insufficient to yield decreased viability observed in STK11KO EOC spheroids. Therefore, we speculate that DUSP4 is at least one protein mediating the reduced metastatic potential due to LKB1 loss in our in vitro spheroid model and potentially decreased tumor formation and metastasis in vivo.

**Discussion**

Peritoneal metastasis in EOC is often associated with the accumulation of malignant ascites, which contains spheroids that play a key role in disease progression (3). We previously reported that EOC cells require LKB1 to maintain spheroid viability and chemoresistance (8). Given the functional relevance of spheroids in EOC metastasis (3, 5–7), targeting LKB1 or its signaling partners in metastatic EOC may have therapeutic value. Herein, we now present additional evidence that EOC cells require LKB1 for maximal metastatic potential and add to the body of literature indicating that LKB1 can function in a pro-oncogenic capacity to promote disease progression. On the basis of our findings, and those of others, we propose that LKB1- or its AMPK-independent signaling partners may represent therapeutic vulnerabilities in metastatic EOC.

In the context of cancer, LKB1 has been commonly described as possessing tumor suppressor-like function. STK11 is frequently deleted in non–small cell lung cancer (NSCLC) (40), and somatic nonsense mutations in STK11 occur with high frequency in primary lung carcinomas and lung cancer cell lines (12), as well as in 20% of cervical cancer (13) and melanoma (11). Loss of LKB1 in the ovarian surface epithelium has been linked to papillary serous ovarian cancer in a mouse model (18). LKB1 protein expression is decreased in HGSOC relative to normal FTE tissue; however, this study also found that genomic loss of both STK11 alleles was associated with improved patient survival (15). In fact, our previous report (8) demonstrated that LKB1 expression is maintained in late-stage EOC and is required for cell survival in spheroids and promotes platinum resistance. Given that FTE cells are the most likely cell-of-origin in HGSOC, we had the direct ability to determine whether LKB1 may act as a tumor suppressor in FT190 cells with its loss resulting in their oncogenic transformation. However, we found that neither cell proliferation, nor colony formation, nor anchorage independence, nor spheroid viability and growth were increased due to STK11 gene inactivation. Because FT190 cells are immortalized by expression of SV40 large T antigen (24), our results also indicate that the combination of p53 and pRb inactivation with LKB1 loss is insufficient for malignant transformation of FTE cells. Given the report suggesting decreased LKB1 protein expression in premalignant STIC lesions and primary serous ovarian tumors (15), we propose that LKB1 reexpression and its prometastatic effects are manifested at later steps in ovarian tumorigenesis after initial neoplastic transformation.

A growing body of evidence suggests that LKB1 can function in context-dependent, protumorigenic capacities. In line with its role in metabolism, deficiencies in LKB1–AMPK signaling sensitize some cancer cells to energy stress. LKB1-deficient NSCLC cells are more sensitive to the mitochondrial complex I inhibitor phenformin compared with NSCLC cells with intact LKB1 (41), and AMPK is required in A549 lung cancer cells for survival under glucose-deprived culture conditions (20). However, LKB1 may also promote oncogenic potential in ways that are not directly linked to the energy stress response.

496 Mol Cancer Res; 18(3) March 2020 MOLECULAR CANCER RESEARCH
Figure 6.
DUSP4 expression is increased due to sustained LKB1 loss. **A,** Lysates from OVCAR8, OVCAR8-STK11KO, HeyA8, and HeyA8-STK11KO cells grown in adherent and spheroid culture were subjected to RPPA analysis. Data are presented as a heatmap of the log2-transformed fold change in expression comparing STK11KO cells to their respective parental cell controls. DUSP4 expression was consistently increased due to LKB1 loss in every sample analyzed. **B,** Immunoblot analysis confirming DUSP4 expression is increased in all three EOC cell lines lacking LKB1 as compared with their respective controls. **C,** Immunoblot analysis demonstrating increased DUSP4 protein in OVCAR8-STK11KO tumor xenografts as compared with OVCAR8 tumors. LKB1 loss was confirmed, and tubulin used as a loading control. **D,** Densitometric analysis of immunoblots in **C.** Data are presented as the pixel intensity volume for DUSP4 normalized to tubulin with the mean value for OVCAR8 tumors set to 1. Statistical analysis was performed using a two-tailed Student t test (**P** < 0.05). **E,** Knockdown of DUSP4 in HeyA8 and HeyA8-STK11KO cells as confirmed by immunoblot analysis. Knockdown was performed using siGENOME SMARTpool (SP) or individual siGENOME siRNAs against DUSP4 (03 and 05). **F,** Partial restoration of intact spheroid formation in HeyA8-STK11KO cells resulting from DUSP4 knockdown. Scale bar, 250 μm. **G,** Partial rescue of cell viability in HeyA8-STK11KO spheroids due to DUSP4 knockdown as determined by Cell-Titer Glo assay in a 96-well ULA format. Two-way ANOVA and Tukey multiple comparisons test was performed (**P** < 0.0001; *, **P** < 0.01; **P** < 0.05).
Expression of LKB1 protein is associated with poor patient prognosis in hepatocellular carcinoma, and ectopic expression of LKB1 in Hep3B cells increases tumor xenograft growth (42). Similar to our study of EOC cells, STK11 knockdown decreases spheroid formation and clonogenicity of MCF-7 cells, indicating that LKB1 can enhance anchorage independence and replicative potential in breast cancer cells (22). We have not yet elucidated the mechanisms by which LKB1 promotes metastatic potential of EOC cells; however, our data provide additional evidence to support its protumorigenic role in yet another cancer-specific context.

During metastasis, cancer cells must overcome significant hurdles to maintain viability and seed the formation of secondary tumors. Enhanced anoikis resistance is associated with greater aggressiveness and tumorigenicity in in vivo models of EOC (43). We found that LKB1 loss had little effect on EOC cell growth under standard adherent culture conditions, while having a clear negative impact on EOC cell viability in suspension. Our findings imply that LKB1 supports anoikis resistance and anchorage-independence in EOC cells through mechanisms that do not involve AMPK. We previously reported that phosphorylation of AMPKα at Thr172, which is required for its full catalytic activity (44), is induced upon EOC spheroid formation (8). Herein, we show that despite LKB1 loss, phospho-AMPKα levels in FTE and EOC spheroids remain elevated, which implies AMPKα activation occurs independently of LKB1 in our spheroid system. One such alternative kinase is CAMKK2 (36), which has been implicated in cell detachment-induced phosphorylation of AMPKα in several breast cancer cell lines (37). Using STO-69, we provide evidence that AMPKα phosphorylation in EOC cells is largely mediated by CAMKK2. Thus, we conclude that phospho-AMPK is induced via CAMKK2 in EOC spheroids, but that AMPK activity is dispensable for maintaining spheroid cell viability in the context of LKB1 activity.

While AMPKα is often described as the primary cell stress signaling mediator downstream of LKB1, we demonstrate that essential LKB1 functions in EOC metastasis are AMPK-independent. LKB1 is known to phosphorylate at least 12 substrates that are similar in structure to AMPK, collectively termed AMPK-related kinases. Broadly, these kinases can regulate cell polarity, migration, and metabolism in specific cell contexts (45). Salt-inducible kinases SIK2 and SIK3 may be required in NSCLC metastasis, but their dependence on LKB1 in this disease is unknown (46–48). AMPK-related kinase ARKS/NUA1 has been reported to be overexpressed in EOC and implicated in the regulation of EMT (49). In line with this, NUA1 expression in EOC is associated with poor patient prognosis (50). Our group is currently pursuing additional NUA1 functions as a substrate of LKB1 in metastatic EOC. Elucidation of these downstream mediators will likely be of clinical relevance because they may represent more specific and suitable therapeutic targets in metastatic EOC than LKB1 itself.

We postulate that EOC cells are adaptively reprogrammed to handle abrogated stress signaling due to sustained LKB1 loss. DUSP4 is commonly increased in NSCLC tumors possessing STK11 loss-of-function mutations. Kaufman and colleagues (51) identified an upregulated 16-gene expression signature, which included DUSP4, in LKB1-deficient NSCLC cells likely due to an altered oxidative stress response mediated by NRF2. We postulate that there is a similar dysregulated response to oxidative stress and hypoxia in the absence of LKB1 function in EOC spheroids and tumor xenografts. This could explain our observation of elevated hypoxia in OVCAR8-STK11KO xenograft tumors correlating with increased DUSP4 expression, perhaps resulting from chronic stress due to LKB1 loss in this cell line. It would be intriguing to study whether LKB1 signaling is required in response to tumor hypoxia and oxidative stress to regulate a downstream transcriptional network and mitigate these key metabolic stresses in cancer. Our results also demonstrate that DUSP4 dysregulation on its own is unable to recapitulate the EOC spheroid phenotype due to LKB1 loss. DUSP4 knockdown partially restored spheroid cell viability, yet its ectopic overexpression had no effect. Interestingly, DUSP4 knockdown rescued cell viability in HeyA8 spheroids, yet the size of the resultant spheroids appeared reduced. This could implicate other as of yet unidentified mediators downstream from LKB1 to affect EOC spheroid growth potential.

While LKB1 may act as a tumor suppressor in other cancers, we have provided additional evidence that LKB1 clearly possesses important functions in a prometastatic capacity during EOC progression. Consistent with the established notion that three-dimensional spheroids contribute to EOC dissemination, we have demonstrated that intact LKB1 is required in both in vitro spheroid and in vivo xenograft models of metastatic disease. Overall, we provide supportive evidence that LKB1 or its direct signaling partners represent viable therapeutic targets in metastatic EOC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A. Buensuceso  
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