JAK2/STAT and LKB1: an interaction linking PRL-mediated metabolic changes and structural integrity in MDA-MB-231 human breast cancer cells

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Janus-activated kinase 2 (JAK2)/Signal transducer and activator of transcription (STAT) signaling is activated by prolactin (PRL) in breast cells and tissue. We have shown previously that PRL affects cellular metabolism in a human breast cancer cell type-dependent manner through differential activation of the Adenosine 5'-monophosphate-activated protein kinase (AMPK) pathway, a response mediated by Liver kinase B1 (LKB1). LKB1 regulates diverse cellular processes, including energy sensing and polarity. We have demonstrated that PRL regulates the expression of LKB1 through STAT proteins in the triple-negative, mesenchymal-like, aggressive MDA-MB-231 breast cancer cell line, and that knock-down of LKB1 dramatically alters the morphology of these cells. In ongoing research, we have established a novel interaction between JAK2 and LKB1. The association between these two proteins is blocked by treating cells with (E)-3-(6-bromopyridin-2-yl)-2-cyano-N-((S0-1-phenylethyl)acylamide) (WP1066), an AG490 analogue that efficiently degrades JAK2. We have also evaluated whether inhibiting sustained PRL signaling through JAK2 affects LKB1-AMPK pathway activation. PRL-induced activation of LKB1, AMPK, and tuberous sclerosis complex 2 (TSC2), and inactivation of acetyl CoA carboxylase (ACC), were abrogated by WP1066 pre-treatment of MDA-MB-231 cells. In contrast, culture of MCF-7 cells in the presence of PRL resulted in sustained phosphorylation of AKT, which is known to block AMPK signaling by directly inactivating TSC2. AKT activation in this particular cell line is likely to be uncoupled from JAK2. As further read-out of the changes induced by blocking JAK2, phosphorylation/activation of STAT3 and STAT5 were also differentially affected by WP1066 pre-treatment in 184B5, MCF-7, and MDA-MB-231 cells. The association between JAK2 and LKB1, the metabolic effects linked to JAK2/LKB1-mediated signaling elicited by PRL in MDA-MB-231 cells, and interplay between several important intracellular signaling networks that regulate both metabolism and cell polarity merit further investigation in an extended panel of cells, as well as patient-derived samples. Our findings are of relevance in gaining a better understanding of the epithelial-to-mesenchymal transition (EMT) and mechanisms that protect aggressive cancer cells, allowing them to adapt to a changing microenvironment during disease progression, which may depend on extracellular cues that are spatio-temporally present.


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Introduction

Serine/threonine kinase 11 (STK11), commonly known as Liver kinase 1 (LKB1), is an important multi-tasking cellular mediator that activates Adenosine 5'-monophosphate-activated kinase (AMPK), as well as a group of other AMPK-related kinases [1]. LKB1 has the unique function of eliciting sustained, adaptive AMPK activation in response to energy stress, serving as a key metabolic checkpoint [2]. LKB1-mediated activation of AMPK restricts cellular growth and proliferation when nutrient supplies are limited or energy demands are high, up-regulating glucose uptake or stimulating catabolism via fatty acid oxidation and glycolysis to replenish cellular Adenosine triphosphate (ATP) reserves. Several phenomena commonly observed in cancer cells, including glucose deprivation [3–4], hypoxia [5], oxidative stress [6], and exposure to nitric oxide [7], as well as fluctuations in levels of extracellular growth factors/hormones and dietary changes [8], may contribute to elevated AMP levels that are sensed by AMPK. In addition to maintaining metabolic homeostasis, activation of AMPK and AMPK-related kinases by LKB1 contributes to cell polarity and cytoskeletal integrity, thereby inhibiting unchecked cellular growth and tumor expansion [9]. The genes encoding *H. sapiens* LKB1 and several Microtubule affinity-regulating kinase (MARK) proteins are homologous to *D. melanogaster* lk1 and Protease-activated receptor 1 (par1) [10] and *C. elegans* PAR-4 and PAR-1 [11], which in these latter two species are involved in establishing the anterior-posterior axis during embryonic development. It was found that the induced expression of STE20-related kinase adaptor protein (STRAD), which complexes with and initiates the activation of LKB1, plays a key role in remodelling the actin cytoskeleton in human intestinal epithelial cells [12]. In addition, it has been shown that by phosphorylating PAR-1, LKB1 contributes to the formation of cellular junctions and controls WNT pathway activation. WNT initiates β-catenin signaling to elicit changes in target gene transcription (canonical), as well as β-catenin-independent (non-canonical) specification of planar cell polarity, thereby affecting the cytoskeleton and cell shape (reviewed in [9]). PAR-1 positively regulates the canonical WNT pathway, inhibiting non-canonical signaling [13], although it is unclear whether WNT up-regulates PAR-1 kinase activity directly or through LKB1. Importantly, studies have shown that LKB1 may control cell polarity primarily through the activation of AMPK rather than through PAR-1, supported by the finding that in LKB1-null *D. melanogaster* epithelial cells, constitutive AMPK activation restored defects in polarity (reviewed in [9]). Based on the existing literature, loss of LKB1 is intricately linked with defects in cellular energy sensing and polarity, facilitating malignant cell proliferation and, potentially, aberrant communication with the extracellular microenvironment, culminating in metastasis.

The control of LKB1 expression by PRL, steroid hormones, and, possibly, cytokines may be of particular relevance in more aggressive breast cancer cells, as LKB1 promotes survival when metabolic conditions are unfavorable [14–16]. We previously established that PRL up-regulates catabolic pathways by activating AMPK in MDA-MB-231 cells, with LKB1 acting as its primary upstream phosphorylator [17]. In addition, we have shown that sustained treatment with prolactin (PRL) transcriptionally up-regulates LKB1 through Janus-activated kinase 2 (JAK2)-Signal transducer and activator of transcription (STAT) signaling, thereby increasing LKB1 expression in MDA-MB-231 human breast cancer cells [18]. This particular cell line represents an aggressive, triple-negative, mesenchymal-like (basal) cell type that is able to metastasize to bone, brain, lung, and the adrenal glands [19, 20]. We demonstrated binding of STAT3 and, more robustly, STAT5 to a Gamma interferon (γ-IFN)-activated sequence (GAS) motif within the distal human LKB1 promoter region in response to sustained PRL treatment [18]. Further computational analysis of the promoter revealed the presence of a putative Hypoxia-inducible factor 1α (HIF1α) site that could confer responsiveness to oxygen deprivation. A conserved Activator protein 1 (AP-1) site is also present in the LKB1 promoter, which may be responsive to stress signaling. The presence of this latter motif is of future interest in assessing STAT-mediated transcriptional changes affecting LKB1 expression, given that others have shown STAT5 activation to inhibit PRL-induced AP-1 activity in a breast cancer cell type-dependent manner [21], and LKB1 is differentially affected by PRL in different breast cancer cells. Also of relevance, the cellular estrogen receptor (ER) status may alter LKB1 promoter responsiveness [18, 22, 23].

Several signaling modules that are activated in the normal and cancerous breast by diverse extracellular stimuli involve the cytoplasmic tyrosine kinase JAK2 (reviewed in [24–26]). JAK2 provides binding sites for and activation of various STAT family members (reviewed in [25, 26]). MAPK [27], insulin receptor substrates [28], focal adhesion kinase (FAK) [29], and phosphoinositide-3-kinase (PI3K)/AKT [30], which are differentially activated in human breast cancer cells [31]. One of four known JAKs, JAK2 is autophosphorylated in response to ligand binding and receptor activation, mediating signaling of the type I cytokine (LepR, LIFR), also including GM-CSF (IL-3R, IL-5R, and GM-CSF-R), gp130 (IL-6R), and single chain (EpoR, TpoR, GHR, and the prolactin receptor; PRLR) family members, as well as the type II cytokine (IFN-R) receptors [32]. Both PRL and PRLR are expressed in the epithelium of the normal and cancerous breast [33], with PRL promoting the proliferation, survival, and migration of malignant cells (reviewed in [25, 34]), also contributing to the
resistance of breast cancer cells to chemotherapeutic agents (reviewed in [35]), acting as a pro-angiogenic factor in normal and malignant mammary tissue [36], and gaining attention as a metabolic hormone (reviewed in [37–40]). We verified that the fully functional PRLR is expressed in representative epithelial-like (luminal) and mesenchymal-like (basal) human breast cancer cells [18]. Establishing a connection between JAK2 and LKB1 would provide a mechanism by which extracellular ligands, including PRL, directly activate the AMPK pathway in breast cancer cells. Positive feedback could stem from JAK2/STAT-mediated transcriptional regulation of LKB1 expression, affecting the cytoskeleton, cell polarity, and the metastatic potential of aggressive, metabolically stressed cancer cells. This latter possibility is particularly relevant given that PRL and JAK2-mediated signaling play essential roles during the epithelial-mesenchymal transition (EMT) in MDA-MB-231 cells, suppressing both their mesenchymal properties and invasive potential [41]. Blocking autocrine PRL signaling in epithelial-like T47D breast cancer cells induces a mesenchymal-like phenotype, also enhancing their invasive properties [41]. We have shown that loss of LKB1 induces dramatic phenotypic changes in MDA-MB-231 cells, altering their typical spindle shape to a more rounded morphology [18]. Loss of LKB1 also affects the expression of β-catenin [18], indicative of a possible EMT. Interestingly, others have shown that knock-down of WNT in MDA-MB-231 cells produces morphological changes similar to those observed upon knock-down of LKB1 [42]. By blocking signaling through JAK2 and STAT protein family members including STAT3 and STAT5, we have aimed to gain a better understanding of (1) the complex mechanisms underlying hormone-responsive LKB1-AMPK pathway activation, (2) the functional significance of differential activation in human breast cancer cells, and (3) the molecular mechanisms underlying the changes in LKB1 expression following sustained PRL treatment.

### Materials and methods

#### Cell Culture

Human cell lines were purchased from ATCC and used in accordance with institutional biosafety guidelines. The immortalized normal human breast epithelial cell line 184B5 was cultured in MEGM media with the recommended supplements (Clonetics), and low-passage (corresponding to less than 20 trypsinizations) MCF-7 and MDA-MB-231 human breast cancer cells were maintained in DMEM, High Glucose (Invitrogen) with 10% fetal bovine serum at 37°C in 5% CO₂. Cells were seeded into 6-well plates (Falcon) at 2.5x10^5 cells per well and allowed to attach overnight. Media was replaced with fresh, complete culture medium appropriate to each cell line. Cells were cultured for 24 hr either in the presence of vehicle (sterile water) or 100 ng/ml of recombinant human PRL (Cedarlane), or pre-treated for 1 hr with 5 µM of the JAK2 inhibitor (E)-3(6-bromopyridin-2-yl)-2-cyano-N-((S0-1-phenylethyl)acrylamide) (WP1066) (Sigma) prior to the addition of PRL for an additional 24 hr.

#### Western Blotting

Western blotting was performed as described previously [17, 18, 22]. Blots were incubated in 1:1000 diluted primary antibody overnight (Table 1) and subsequently exposed to the appropriate anti-rabbit or anti-mouse IgG horseradish peroxidase secondary antibody (1:3000, Cell Signaling Technology). Membranes were stripped and reprobed with anti-Actin primary antibody.

#### Co-Immunoprecipitations

Co-immunoprecipitations (IPs) with total JAK2 or total LKB1 antibody were carried out as described previously [18]. Normal rabbit IgG (SC-2027; Santa Cruz

### Table 1. List of antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone, Species</th>
<th>Supplier, Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>C4; Mouse mAb</td>
<td>MP Biomedicals, LLC #691001</td>
</tr>
<tr>
<td>phos-ACC (Ser79)</td>
<td>Rabbit pAb</td>
<td>Cell Signaling Technology #3661</td>
</tr>
<tr>
<td>ACC1/2</td>
<td>C83B10; Rabbit mAb</td>
<td>Cell Signaling Technology #3676</td>
</tr>
<tr>
<td>phos-AMPKα (Thr172)</td>
<td>40F9; Rabbit mAb</td>
<td>Cell Signaling Technology #2535</td>
</tr>
<tr>
<td>AMPKα</td>
<td>23A3; Rabbit mAb</td>
<td>Cell Signaling Technology #2603</td>
</tr>
<tr>
<td>phos-AKT (Ser473)</td>
<td>73E1; Rabbit mAb</td>
<td>Cell Signaling Technology #3787</td>
</tr>
<tr>
<td>phos-AKT (Thr308)</td>
<td>244F9; Rabbit mAb</td>
<td>Cell Signaling Technology #4056</td>
</tr>
<tr>
<td>AKT (pan)</td>
<td>11E7; Rabbit mAb</td>
<td>Cell Signaling Technology #4685</td>
</tr>
<tr>
<td>JAK2</td>
<td>D2E12; Rabbit mAb</td>
<td>Cell Signaling Technology #3230</td>
</tr>
<tr>
<td>phos-LKB1 (Ser428)</td>
<td>C67A3; Rabbit mAb</td>
<td>Cell Signaling Technology #3482</td>
</tr>
<tr>
<td>LKB1</td>
<td>D60C5; Rabbit mAb</td>
<td>Cell Signaling Technology #3047</td>
</tr>
<tr>
<td>phos-STAT3 (Tyr705)</td>
<td>D3A7; Rabbit mAb</td>
<td>Cell Signaling Technology #9145</td>
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<td>79D7; Rabbit mAb</td>
<td>Cell Signaling Technology #4904</td>
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<td>phos-STAT5 (Tyr694)</td>
<td>Rabbit pAb</td>
<td>Cell Signaling Technology #9351</td>
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<tr>
<td>phos-TSC2 (Ser1387)</td>
<td>Rabbit pAb</td>
<td>Cell Signaling Technology #5584</td>
</tr>
<tr>
<td>TSC2</td>
<td>Rabbit pAb</td>
<td>Cell Signaling Technology #3612</td>
</tr>
</tbody>
</table>
Biotechnology, Inc.) was included as a negative control in a representative IP reaction, and a positive control (input) representing approximately 5-10% of cleared lysate was included during Western blotting.

Densitometry and Statistical Analyses

Image J analysis software was used to densitometrically analyze Western blots for changes in the levels of total and phosphorylated proteins relative to untreated controls within a treatment group. Values were corrected for actin or total protein/actin, respectively. Using GraphPad Prism analysis software, results representing the mean ± SEM of at least three independent experiments were analyzed by t-test to determine differences between groups, which were considered to be statistically significant at p < 0.05, indicated by a star (*).

Results and discussion

LKB1 is known to interact and form a complex with STRAD (also known as LYK5) and Mouse protein 25 (MO25), thereby directly activating AMPK by phosphorylating its α subunit at Thr172 [43]. Other proteins that interact with LKB1 include FYN [44], CDC37 [45], HSP90AA1 [45], and SMARCA4 (also known as BRG1) [46]. Interestingly, JAK2 physically associates with FYN [47], as well as HSP90 [48]. HSP90 forms a complex with CDC37 [49], and, using a proteomics approach, STAT5 and STAT3 have been shown to complex with HSP90 [50]. SMARCA4 regulates transcription by altering the chromatin structure associated with specific genes, also interacting with STAT2 to control IFN-α-mediated gene expression [51]. In the mammary gland, sustained activation of STAT5 is required for chromatin remodeling and cell polarity [52], and JAK2 also associates with chromatin [53]. These commonalities support the possibility that JAK2 and/or STAT proteins complex with LKB1 in human breast cancer cells. Based on a computational analysis using PIPs Human Protein-Protein Interaction Prediction software (www.compbio.dundee.ac.uk) [54, 55], we determined that LKB1 was among the proteins predicted to potentially associate with JAK2 (IPI00031016), with a putative interaction score of 26.8. While this score is low compared to, for example, the well-known JAK2 interactors STAT5A (2.39E5) and STAT3 (299.0), it warranted further investigation, especially given that Suppressor of cytokine signaling 1 (SOCS1), which has experimentally been shown to physically interact with JAK2 [56, 57], had a low...
predicted interaction score (40.50). Co-immunoprecipitations revealed that total LKB1 basally associates with total JAK2 in 184B5, MCF-7, and MDA-MB-231 cells, and that this interaction could be disrupted.
by pre-treating cells with WP1066 (Figure 1A and B). WP1066 is an analogue of the JAK2 inhibitor AG490 that effectively degrades JAK2 protein and has also emerged as a blocker of STAT3 activation\textsuperscript{[58]}. It may seem paradoxical that LKB1, typically classified as a tumor suppressor (reviewed in\textsuperscript{[59]}), interacts with or may be part of a protein complex that includes JAK2, and is also phosphorylated in response to sustained PRL treatment in MDA-MB-231 cells. Clearly, it is important to evaluate the role of LKB1 in a cell type-dependent context, as the severity of stress experienced by different cancer cells due to a changing microenvironment may uniquely drive their

Figure 3. The effect of blocking JAK2 on PRL-mediated phosphorylation of AKT. (A) Representative Western blots depicting phosphorylated (p-) AKT at both Ser473 and Thr308 and total AKT in 184B5, MCF-7, and MDA-MB-231 cells. (B) Graphical representation of the densitometric analyses representing at least 3 independent experiments. -: untreated, +: 100 ng/ml of PRL for 24 hr, ++: 1 hr pre-treatment with 5 µM WP1066 prior to adding PRL.
adaptive responses, ensuring continued survival and further proliferation \[15, 16\]. This is particularly relevant given that, under normal metabolic conditions, LKB1 induces apoptosis, while it is thought that during energy stress, a phenomenon common to aggressive cancer cells, LKB1-AMPK signaling protects cells until aberrant AMP/ATP ratios are stabilized \[16\]. Indeed, the LKB1-AMPK pathway initiates survival mechanisms during growth factor

Figure 4. Blocking JAK2 distinctly affects levels of phosphorylated STAT3 and STAT5 in a cell type-dependent manner. Representative Western blots depicting the effect of blocking JAK2 on levels of phosphorylated (p-) and total STAT5 and STAT3 in 184B5, MCF-7, and MDA-MB-231 cells. (B) Graphical representation of the densitometric analyses representing at least 3 independent experiments. +: 100 ng/ml of PRL for 24 hr, ++: 1 hr pre-treatment with 5 µM WP1066 prior to adding PRL.
withdrawal by regulating p27kip1, providing cancer cells a potential means to overcome energetically unfavorable conditions.\(^{14}\)

In order to better understand the relationship between PRL, JAK2, and LKB1-AMPK pathway activation, we pre-treated representative breast cancer cells with WP1066. In MDA-MB-231 cells, levels of phosphorylated LKB1, AMPK\(\alpha\), Acetyl CoA carboxylase (ACC), and Tuberous sclerosis complex 2 (TSC2), an important tumor suppressor\(^{60}\) that is phosphorylated by AMPK at Ser1387\(^{61}\), decreased significantly after WP1066 pre-treatment (Figure 2A and B). In contrast, the LKB1-AMPK pathway was not significantly affected by WP1066 in MCF-7 cells (Figure 2A and B). AMPK activation is blocked by AKT\(^{62}\), and the inhibition of the mammalian Target of rapamycin (mTOR) by TSC2, a response that has been linked with protecting cells from apoptosis during energy deprivation, may be lifted by AKT through its direct phosphorylation and inactivation of TSC2 at its Thr1462 residue\(^{61,63,64}\). Akt is required for murine Prlr-Jak-Stat5 signaling in normal breast epithelial cells\(^{65}\), and in pregnant mice, Akt drives terminal mammary epithelial cell differentiation, with its loss reducing the production of lactose, fat, and protein, the three main milk constituents\(^{65}\). WP1066 pre-treatment did not significantly alter levels of phosphorylated AKT at either its Ser or Thr residues in MCF-7 cells, suggesting that activation of AKT by PRL is likely to be uncoupled from JAK2 in this cell line (Figure 3A and B). This is consistent with PRL mediating its signal through the non-receptor protein tyrosine kinase SRC in

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Figure 5. Schematic diagram of the potential relationship between JAK2, LKB1, energy sensing, and cell polarity/cytoskeletal integrity. Sustained treatment of MDA-MB-231 cells, in which JAK2 and LKB1 are basally associated, induces activation of the LKB1-AMPK pathway. The roles of LKB1 in energy sensing and cell polarity are linked with the phosphorylation (p) of serine and threonine residues on various AMPK-related kinases, including AMPK and PAR-1. AMPK directly phosphorylates TSC2, thereby inhibiting mTOR activity, which is directly opposed by activated AKT. PAR-1 contributes to the formation of cellular junctions, as well as canonical WNT pathway activation. PRL-mediated signaling through JAK2/STATs up-regulates LKB1 expression through binding of STAT proteins to a GAS site in the promoter region, thereby increasing LKB1 protein levels.
MCF-7 cells [31]. We found that significant increases in Ser1387 phosphorylation/activation of TSC2 in response to sustained PRL treatment occurred only in MDA-MB-231 cells, in which LKB1 and AMPK were also significantly activated (Figure 2A and B). Interestingly, induction of TSC2 by AMPK and its negative regulation of mTOR have been linked with non-canonical WNT signaling, providing a potential link between energy signaling and cell polarity [66].

One of the key proteins activated by PRL through JAK2 is STAT5, which controls the expression of genes associated with the cell cycle (reviewed in [35]) and milk protein synthesis [67,68]. In contrast to changes that occur in mammary epithelial cells during the onset of lactation, PRL inhibits lipogenesis in murine adipocytes through Stat5a by blocking the transcriptional activation of Fatty acid synthase (FASN), a key regulatory enzyme [69]. PRL-mediated activation of STAT5 may promote the anti-invasive behavior of human breast cancer cells, assessed by examining changes in cell scattering, matrix metalloproteinase secretion, and cell invasion through Matrigel [70]. In ER-positive T47D and ZR-75-1 cells, and in the less well-differentiated, ER-negative BT-20 cell line, a 24 hour treatment with PRL stimulated cell surface accumulation of E-cadherin [70]. In marked contrast, tumors derived from T47D-injected nude mice over-expressing dominant negative Stat5a were smaller due to increased apoptosis [71]. STAT5 may act as an activator or repressor of transcription, depending on the cell type in which it is expressed, its binding partners, whether it is present as a dimer or tetramer, and which isoform is predominant (reviewed in [72]). Similar to STAT5, the functional effects of STAT3 are diverse and seemingly contradictory. The JAK2/STAT3 signaling pathway is preferentially activated in response to Interleukin 6 (IL-6) in mesenchymal-like breast cancer cells including MDA-MB-231 cells, but not in epithelial-like MCF-7 and T47D cells [73]. PRL is also able to activate STAT3 through JAK2 [74]. In mice with a conditional Stat3 knockout, suppressed epithelial cell apoptosis and delayed mammary gland involution have been reported [75]. However, STAT3 is constitutively active in many breast cancer cells, supports proliferation, and its inhibition reduces tumor formation [76-80]. STAT3 activation up-regulates the expression of pro-survival genes including B-cell lymphoma 2 (BCL2), BCL-X, and Survivin [81,82]. In the murine liver, Stat3 deletion is linked with the expression of genes involved in gluconeogenesis and insulin resistance, and hepatic Stat3 signaling may be required for normal glucose homeostasis [83]. Stat3 regulates genes involved in murine alveolar epithelial cell survival and surfactant/lipid synthesis [84], while in the liver, Stat3 negatively regulates fatty acid anabolism [85]. STAT3 and STAT5A may potentially associate with each other based on a predicted PIPs interaction score of 2.89E3, and it has been reported that a complex activated by Colony stimulating factor 1 and Platelet-derived growth factor may contain STAT3/STAT5 heterodimers that interact with DNA [86]. Interestingly, there is evidence supporting an active role for basal, unphosphorylated STAT5s in regulating gene expression (reviewed in [87]). Total STAT3 induces and co-regulates transcription in the nucleus [88], also acting as a cytoplasmic stabilizer of microtubules [89]. We determined basal levels of phosphorylated STAT5 to be nearly undetectable in MDA-MB-231 cells, which is reflective of aggressive, node-positive breast cancers expressing significantly lower levels of activated STAT5 compared to non-metastatic tumors [90]. However, also consistent with reports by others [21], total levels of STAT5 were robustly present in MDA-MB-231 cells, and sustained PRL treatment induced slight but consistent increases in STAT5 phosphorylation [18]. Also consistent with other studies [91], constitutively high levels of activated STAT3 were observed in MDA-MB-231 cells, with PRL inducing slight further increases in its sustained phosphorylation [18].

Pre-treatment with WP1066 significantly decreased the levels of activated STAT3, concomitant with an increase in phosphorylated levels of STAT5 (Figure 4A and B). The inverse occurred in MCF-7 cells, with levels of phospho-STAT5 decreasing as phospho-STAT3 increased (Figure 4A and B). Our findings suggest that STAT family members influence LKB1-AMPK pathway activation and regulation of LKB1 expression in a cell type-dependent manner and may functionally compensate for one another. In support of the notion of (a) compensatory mechanism(s), deleting Stat3 in murine pulmonary cells up-regulates the expression of genes associated with JAK-STAT, including JAK1 and JAK2 [84], facilitating signaling through other STAT family members. In future experiments, we aim to investigate whether over-expressing different STAT proteins, particularly STAT3, STAT5A, and STAT5B, in MDA-MB-231 cells produce similar effects on LKB1 promoter activity as does sustained treatment with PRL.

Although a greater number of breast cancer cell lines will need to be examined before making broadly applicable conclusions, we propose that PRL selectively initiates what could be referred to as a cellular protective or coping mechanism. This adaptive response may be particularly relevant in subsets of aggressive, mesenchymal-like breast cancer cells, arising due to changes in the availability of nutrients and oxygen sensed by AMPK. Although it remains to be experimentally determined, activation of LKB1-AMPK-TSC2 by PRL may ultimately protect cells from apoptosis. In support of this notion, PRL does not induce any significant changes in the LKB1-AMPK pathway in 184B5 normal breast epithelial cells, which are not under metabolic stress. In low oxygen, low glucose conditions, cellular oxidative phosphorylation shifts to anaerobic metabolism. Important parallels can be drawn...
between energy stress in aggressive cancer cells and myocardial ischemia, in which the initiation of glycolysis has been attributed to AMPK-mediated phosphorylation/activation of Phospho-fructose kinase 2 (PFK2), leading to the inactivation of several key enzymes associated with energy-consuming biosynthetic pathways [5]. In contrast, in epithelial-like SKBR3 human breast cancer cells [73], PRL up-regulates oxidative phosphorylation through STAT5 [92]. In MDA-MB-231 cells, PRL induces signaling pathways and elicits metabolic effects that are compensatory and distinct from those observed in less aggressive cells, and it will be of interest to examine whether LKB1-AMPK pathway activation is linked with changes in the activity of PFK2 and other relevant enzymes in this and other triple-negative cell lines.

As already mentioned, the activation of LKB1 is also associated with cell polarity and reorganization of the actin cytoskeleton, as well as the formation of gap junctions in the absence of cell-cell contacts [12]. In motile non-small cell lung cancer cells, LKB1 colocalizes with the serine/threonine CDC42 and p21-activated kinase 1 (PAK1) [93], thereby regulating cell morphology and motility. PAK proteins link the RHO family of GTPases to both reorganization of the cytoskeleton and nuclear signal transduction, and are targeted by CDC42 and RAC, two GTP binding proteins [84]. In relation to breast cancer, increased migration of T47D cells has been linked with PAK1 [95], which acts as a substrate for JAK2 and is phosphorylated in response to PRL [96, 97]. JAK2-activated PAK1 in turn phosphorylates the actin-binding protein Filamin A, inducing PRL-mediated cell migration [95]. Conversely, it has been shown that by activating JAK2, PRL regulates the morphogenic program of MDA-MB-231 cells, suppressing their metastatic potential and in effect acting as an invasion suppressor [41]. In addition to activating AMPK, LKB1 also phosphorylates and inactivates PAK1, suppressing cell migration [98]. STAT5A is predicted to interact with another PAK family member, PAK4 (based on a PIPs score of 22.2), and blocking JAK signaling using a novel inhibitor, Oclacinib, inhibits PAK4 kinase activity [99]. Interestingly, Oclacinib also blocks the activity of the STE20-like kinase STK24 (also known as MST2, a protein in the same family as STRAD). It is important to also keep in mind that AMPK, directly and through its activation of TSC2/inactivation of mTOR, is itself intricately involved not only in metabolism, but in the regulation of cell size and shape (reviewed in [9]). While the molecular mechanisms that underlie the effects of PRL on LKB1-AMPK remain to be defined, we suggest that in MDA-MB-231 cells, the direct association between JAK2 and LKB1, as well as its phosphorylation and activation in response to PRL treatment, “put the brakes” on cell migration typically promoted by PAK1 and mTOR activation until these cells reach an energetically favorable state to metastasize. Interestingly, it has been shown that cytoskeletal integrity is required to effectively transduce signals between PRLR and STAT5 [100]. In metastatic breast cancer cells that have entered the circulation, levels of activated STAT5 are very low [90], but could potentially be up-regulated once malignant cells infiltrate a new site. Unlike its rapid phosphorylation in T47D and MCF-7 cells, STAT5 is only activated by PRL in MDA-MB-231 cells after sustained treatment. It is possible that, following gradual increases in levels of phosphorylated STAT5, actin networks and microtubules are re-established. WNT signaling is also implicated in breast cancer cell metastasis. WNT pathway activation induces EMT, particularly during mammary gland development [101]. Epithelial cells transform into mesenchymal cells by detaching from laminin, a process that involves the down-regulation of cadherins. Repressing WNT/β-catenin signaling prevents EMT and inhibits lung metastasis of basal-like breast cancer cells [102]. Interestingly, WNT signaling inhibits TSC2 phosphorylation by activating mTOR [60], providing another link between energy sensing and cell polarity, with LKB1 modulating both. It will be of considerable interest to examine the possible relationship between PRL, LKB1-AMPK, and the WNT pathway in an extended panel of cells, as the molecular signature of different breast cancer cells is central to these processes. We have summarized some of the possible connections in Figure 5.

Conclusions

Our findings provide novel links between PRL, JAK2, STAT proteins, LKB1, and downstream pathways including the activation of AMPK, which may be of particular importance in aggressive, metabolically stressed cancer cells. This signaling module is initiated in a breast cancer cell type-dependent manner in response to metabolic cues related to the cellular energy status. Activation of LKB1-AMPK likely protects metabolically stressed cells until AMP/ATP ratios return to normal, either through PRL or a secondary pathway that is induced by PRL and also involves JAK2. While LKB1 associates with JAK2 in normal breast epithelial cells and representative breast cancer cell lines, blocking JAK2 evokes distinct downstream differences in AMPK pathway activation, suggesting the involvement of a unique signaling component that comes into play in specific breast cancer cells. The in vitro findings reported here should therefore be assessed in a large panel of patient-derived primary and metastatic tumor samples. The association of JAK2 with LKB1, which potentially supports metabolic signaling and cytoskeletal rearrangements, as well as PRL-mediated regulation of LKB1 expression by STATs, merit further investigation in the context of cross-talk with other growth factors and hormones. Establishing whether cytokines such
as IL-6 that also signal through JAK2 associate with or affect the expression of LKB1 will provide valuable mechanistic insights into the progression of other cancers and diseases such as diabetes.

Conflicting interests
The authors declare that they have no Conflicting interests.

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