Caveolin-1–LRP6 Signaling Module Stimulates Aerobic Glycolysis in Prostate Cancer

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Abstract

Caveolin 1 (Cav-1) is a plasma membrane–associated protein with the capacity to modulate signaling activities in a context-dependent fashion. Interactions between Cav-1 and low-density lipoprotein receptor–related protein 6 (LRP6) were reported to be important for the regulation of Wnt-β-catenin (β-caten) signaling. Cav-1 also interacts with insulin and IGF-I receptors (IGF-IR/IR) and can stimulate IR kinase activities. We found positive correlation between Cav-1 and LRP6 expression in both human primary prostate cancer and metastasis tissues and in PC-3 cells. Cav-1 stimulation of Wnt-β-caten signaling and c-Myc levels was positively associated with LRP6 expression in LNCaP, PC-3, and DU145 prostate cancer cells. Importantly, LRP6 and, to a lesser extent, Cav-1 were found to stimulate aerobic glycolysis. These activities were positively associated with the expression of HK2 and Glut3 and shown to be dependent on Akt signaling by both gene knockdown and chemical inhibition methods. We further showed that Cav-1 and LRP6 exert their effects on Akt and glycolytic activities by stimulating IGF-IR/IR signaling. Overall, our results show that Cav-1 interacts with LRP6 to generate an integrated signaling module that leads to the activation of IGF-IR/IR and results in stimulation of Akt–mTORC1 signaling and aerobic glycolysis in prostate cancer. Cancer Res; 73(6): 1900–11. ©2012 AACR.

Introduction

Caveolin 1 (Cav-1) is a major component of caveolae, and is involved in a variety of cellular processes including, endocytosis, lipid homeostasis, signal transduction, and tumorigenesis (1, 2). Cav-1 interacts with specific proteins through the Cav-1 scaffolding domain and regulates multiple malignancy-associated activities including cellular transformation, tumor growth, angiogenesis, multidrug resistance, cell survival, migration, and metastasis (3). Cav-1 levels are positively correlated with tumor stage and grade in numerous cancer types including prostate cancer (3–8). We have shown that prostate cancer cells secrete biologically active soluble Cav-1 protein, which can promote prostate cancer cell viability through antiapoptotic activities and clonal growth in vitro, similarly to that observed after enforced expression of Cav-1 (9, 10). We have further shown that recombinant Cav-1 protein is taken up by prostate cancer and endothelial cells in vitro and increases angiogenic activities by activating Akt- and/or nitric oxide synthase–mediated signaling (11). Cav-1 was also previously shown to be critical in inducing internalization of low-density lipoprotein receptor–related protein 6 (LRP6) and activating the Wnt–β-caten (β-caten) pathway (12). Wnt ligands bind LRP5 and 6 and Frizzled (FZD) receptors and mediate canonical β-caten signaling in various cell types, including both normal and malignant epithelial and stromal cells (13, 14). Multiple Wnt genes or proteins (both canonical and noncanonical) are upregulated or mutated in primary prostate cancer, osteoblastic prostate cancer bone metastases, and castrate-resistant prostate cancer (15–17).

A key step after Wnt stimulation is the phosphorylation of the LRP6 intracellular domain by casein kinase 1γ. This phosphorylation event activates LRP6 and promotes recruitment of Axin (18, 19), which results in stabilization of β-caten (13). Previous work showed that Wnt3a can induce internalization of the FZD–LRP6 complex through Cav-1 (12). Wnt3a induces the phosphorylation-dependent binding of Axin to LRP6 and the Cav-1–mediated internalization of LRP6 as independent events. The formation of a complex between the cytoplasmic region of LRP6 and Cav-1 is sufficient for the activation of β-caten pathway. Wnt3a possibly triggers the interaction of LRP6 with Cav-1 and promotes the recruitment of Axin to LRP6 after phosphorylation by glycogen synthase kinase 3β (GSK-3β) thereby, Cav-1 inhibits β-caten’s binding to Axin (12). Dkk1, on the other hand, induces internalization of LRP6 in a clathrin-dependent manner and inhibits β-caten activities (20).

Cav-1 also interacts with insulin and insulin-like growth factor I (IGF-I) receptors (IR and IGF-IR) and can stimulate IR

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kinase activities (21–23). Insulin and the IGF family are associated with various malignancies, including prostate (24–26), lung (27), and premenopausal breast cancers (28). Both IR and IGF-IR are commonly expressed in human prostate cancer specimens, implicating insulin and IGF-I signaling in the initiation and/or progression of prostate cancer (29). Insulin’s binding to the IR triggers phosphorylation of a complex network of intracellular effectors involved in glucose metabolism and GLUT4 translocation. The phosphoinositide-3-kinase (PI3K) signaling pathway is considered the main cascade responsible for these metabolic actions of insulin. Although insulin may also elicit mitogenic effects linked mainly to activation of the mitogen-activated protein kinase (MAPK) cascade, PI3K also contributes to IR-mediated cell proliferation and survival. These 2 cascades are interconnected and converge on the mTOR–S6K pathway, a major regulator of cell growth, survival, and metabolism. Although insulin and IGFs play distinct physiologic roles, they use the same signaling pathways, involving PI3K and Akt or Ras and MAPK, which mediate responses to many other cellular stimuli (30). Furthermore, the available data suggest crosstalk between insulin and Wnt signaling in different cells. Insulin was reported to inhibit GSK-3β through the activation of Akt, which stimulates the phosphorylation and inhibition of GSK-3β at serine 9 (31, 32). GSK-3β is also inhibited in response to Wnt stimulation (33). Both insulin and Wnt-stimulated pathways may lead to activation of the mTOR signaling pathway and regulation of translation (34). Insulin and IGF-1 were also reported to stimulate β-catenin accumulation in intestinal L cells (35), and both activated the β-catenin signaling pathway in hepatoma cells (36). In addition, stimulated Wnt–β-catenin signaling in skeletal muscle cells increases insulin sensitivity and increases glucose transport via the activation of Akt and the AMPK signaling pathway (37).

One major difference between cancer cells and normal cells is how they metabolize glucose: most cancer cells primarily metabolize glucose by glycolysis, whereas most normal cells completely catabolize glucose by oxidative phosphorylation (38). This shift to aerobic glycolysis with lactate production, coupled with increased glucose uptake, is likely used by proliferating cells to promote the efficient conversion of glucose into the macromolecules needed for increased cellular mass. Although the selection for cancer cells to convert to aerobic glycolysis remains unclear, glutamine and glucose metabolism, which has been associated with cancer cell growth, may be related to the production of acetyl-CoA and NADPH needed for fatty acid synthesis (39–41). Recently, important oncogenic connections to anaerobic metabolism have been elucidated. PI3K signaling through Akt can stimulate both growth control and glucose metabolism, including increased glucose transporter expression, enhanced glucose capture by hexokinase, and stimulated phosphofructose activity (39). We reasoned that interactions between Cav-1 and LRP6 may alter downstream signaling activities in prostate cancer, and thus alter prostate cancer metabolism.

We show here that Cav-1 knockdown led to reduced levels of total and phosphorylated LRP6 (P-LRP6) in prostate cancer cells under the constitutive expression of Wnt (15–17). Furthermore, Cav-1–LRP6 signaling module activities stimulated IGF-IR/IR activation, leading to Akt–mTORC1–mediated aerobic glycolysis that was independent of β-cat. These results thus have implications for the switch from oxidative phosphorylation in normal cells to aerobic glycolysis in prostate cancer.

Materials and Methods

Cell lines and cultures

LNCaP, PC-3, and DU145 prostate cancer cells were obtained from the American Type Culture Collection and cultured as described previously (9), and were validated by STR DNA fingerprinting with an AmpFISTR Identifier kit (Applied Biosystems) in The University of Texas MD Anderson Cancer Center. Characterized Cell Line Core.

Materials

Recombinant human Wnt3a and Dkk1 were from R&D Systems; Akt1/2 Rapamycin and PPP, from Calbiochem; and AG1024, from Santa Cruz Biotechnology. Recombinant adenoviral vectors AdCav-1 and AdRSV were generated (5, 42) and the phCav-1-V5-His plasmid constructed as described previously (43). The LRP6-Flag Plasmid was constructed as described (Supplementary Data).

RNA interference

Knockdown experiments in PC-3 and LNCaP cells were achieved by transiently transfecting the cells with a pool of Cav-1– or LRP6-specific siRNAs (Invitrogen and Santa Cruz); IR- or IGF-IR–specific siRNAs (Santa Cruz); a pool of non-targeting siRNAs (Invitrogen) was used as a negative control (NCsi). Lipofectamine RNAiMax transfection reagent (Invitrogen) was used. Stable DU145 short hairpin RNA (shRNA) cells were generated by using Cav-1 shRNA plasmid or NCshRNA by using shRNA transfection reagent (Santa Cruz). Stably transfected cells were selected with 1.0 μg/mL puromycin.

Analysis of RNA expression by quantitative reverse-transcription PCR

Total RNA was collected 24 hours after transfection and isolated using a mirVana RNA isolation kit (Ambion, Life Technologies), and reverse transcribed according to manufacturer’s instructions (Applied Biosystems). Relative changes in gene expression were normalized to β-actin RNA in the same cDNA sample.

Human prostate specimens

Specimens from 33 radical prostatectomies and 6 bone biopsies of patients with prostate cancer were obtained from the MD Anderson Cancer Center prostate cancer Specialized Program of Research Excellence tissue core after Institutional Review Board approval. All specimens were processed as described previously (44). Most (27/33, 82%) of these specimens were pathologically staged pT2; 5 others were pT3a, and 1 was pT3b. Sixteen of the 33 specimens (48.5%) had differentiation patterns of Gleason score 6; 13 of Gleason score 7; 3 of Gleason score 8; and 1 of Gleason score 9.
Metastatic lesions were present in all 6 bone biopsy specimens derived from the patients who had been treated with hormonal therapy.

**Cytoplasmic and nuclear fractionation**

Cytoplasmic and nuclear fractions were isolated by using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit from Thermo Scientific.

**Cell proliferation assay**

PC-3 cells in 0.1 mL of medium were placed in 96-well plates at a concentration of 3–5 × 10³ cells per well and transfected with Cav-1–specific siRNA (Ambion) and LRP6–specific siRNA (Santa Cruz) by using Lipofectamine RNAiMax (Invitrogen). Cell proliferation was determined by using an MTS assay (Promega) according to the manufacturer's instructions.

**Glucose uptake and lactate assays**

LNCAp (6 × 10⁵ cells/well) and PC-3 cells (2.7 × 10⁵ cells/well) were cultured on 6-well plates and treated as indicated. For the glucose-uptake assay, cells were incubated in glucose-free RPMI-1640 medium (Invitrogen) for 6 hours and then in medium containing 0.1 mmol/L 2-deoxyglucose and 10 nmol/L [2-¹⁴C]deoxyglucose for 40 minutes. Cells were next washed with cold PBS and lysed by adding 10 mmol/L NaOH containing 0.1% Triton X-100. The lysates were then subjected to liquid scintillation counting for [¹⁴C] level. The total protein concentration per well was used for normalization.

Lactate levels in the culture medium were measured by using an EnzymChrom L-Lactate assay kit (BioAssay Systems) according to the manufacturer's instructions. The total number of cells was used for normalization.

**Immunoprecipitation and Western blot analyses**

Antibodies to Cav-1, c-Myc, and histone H1 were from Santa Cruz. P-LRP6 (S1490), LRP6, P-Akt (S473), P-IR (Y1146)/IGF-IR (Y1131), IR, IGF-IR, S6K, P-S6K (T389), and HK2 antibodies were from Cell Signaling. Other antibodies (α-actin, α-tubulin, polyclonal Flag-tag (Millipore); Akt and α-actin, α-tubulin, polyclonal Flag-tag (Sigma); mouse monoclonal V5-tag (Invitrogen); and polyclonal V5-tag (Genscript)).

UN-SCAN-IT gel analysis software (Silk Scientific) was used for quantification. Data were expressed as the ratios of either phosphorylated protein per total protein or total protein per loading control protein (i.e., α-actin, α-tubulin, or histone H1, as indicated), relative to those in negative (untreated) controls.

**Results**

**Cav-1 and LRP6 interact in prostate cancer**

Immunostaining analysis showed minimal or low levels of Cav-1 and LRP6 in the glandular epithelial cells of tumor-adjacent benign prostate epithelia (Fig. 1). Cav-1+ cancer cells were found in 9 (27%) of the 33 human prostate cancer specimens, and they tended to be focally distributed, as described previously (44). Twenty-two (67%) of the 33 specimens showed LRP6+ cancer cells broadly distributed, with variable expression levels. All 9 Cav-1+ specimens were also LRP6+, whereas 13 (54%) of the 24 Cav-1− cancers were LRP6+ (Fig. 1A). Correlation analysis showed that Cav-1 and LRP6 positivity tended to be positively associated (correlation coefficient r/0.289), but this positive relationship did not achieve statistical significance (P = 0.103).

Because the Cav-1 expression in prostate cancer cells was usually heterogeneous and focal (44), we compared LRP6 expression levels between the Cav-1+ and Cav-1− areas in the 9 Cav-1+ prostate cancer specimens (Fig. 1A). The average LRP6 immunostaining score in the cancer cells of the Cav-1+ areas was statistically significantly higher than it was in the Cav-1− areas of the same prostate cancer (Fig. 1B; P < 0.005). This positive relationship was confined to the epithelial compartment: the LRP6 level in stromal cells was invariably low and independent of the stromal Cav-1 level. Among these prostate cancer specimens, neither Cav-1 nor LRP6 expression correlated with the Gleason score or the pathologic stage. All of the 6 bone metastases specimens evaluated exhibited high expression levels of Cav-1 and LRP6 (Fig. 1A).

We further analyzed the interaction between Cav-1 and LRP6 in PC-3 cells by using quantitative reverse-transcription PCR (qRT-PCR) analysis of RNA expression with Cav-1- and LRP6-specific siRNAs (i.e. Cavsi, LRP6si). Independent Cavsi treatments significantly reduced LRP6 RNA expression, and independent LRP6si treatments significantly reduced Cav-1 RNA expressions, compared with that produced by NCsiRNA treatment (P < 0.0001, all conditions; Fig. 1C). Western blotting also showed that Cav-1 knockdown (2 independent experiments) in PC-3 cells markedly reduced LRP6 levels (60%) and LRP6 knockdown (2 independent experiments) markedly reduced Cav-1 (80%) relative to the levels with NCsi treatment (Fig. 1D).
These data show a close interaction between Cav-1 and LRP6 and suggest that a positive reciprocal mechanism promotes coordinate expression of Cav-1 and LRP6.

Cav-1 stimulates Wnt–β-cat signaling by modulating LRP6 phosphorylation

To study the effect of Cav-1 expression on Wnt–β-cat signaling, we used Cav-1+ LNCaP cells and Cav-1− PC-3 and DU145 cells. AdCav-1 (AdCa)-mediated overexpression of Cav-1 in LNCaP cells led to increased cytoplasmic levels of P-LRP6 in response to Wnt3a, relative to control AdRSV (AdCtl). Cav-1 overexpression also increased the cytoplasmic levels and nuclear translocation of active β-cat and c-Myc in both the absence and presence of Wnt3a (Fig. 2A).

In PC-3 cells, Cav-1 knockdown reduced the cytoplasmic levels of total and P-LRP6; these levels were not restored by Wnt3a treatment. The cytoplasmic and nuclear levels of active β-cat were also reduced markedly (50%–70%) by Cav-1 knockdown, whereas nuclear translocation of c-Myc was reduced (50%) in response to combined Wnt3a and Cav-1 siRNA treatment (Fig. 2B).

The effects of Cav-1 knockdown in DU145 cells were examined by using 2 Cav-1 shRNA stable clones with different Cav-1 levels (Cavsh11 and Cavsh11G). Total and P-LRP6 were reduced (20%–90%) in both clones in proportion to the reductions in Cav-1 levels. In addition, the cytoplasmic and nuclear accumulation of active β-cat, total β-cat, and c-Myc were markedly reduced in both Cavsh11 and Cavsh11G (40%–80%; Fig. 2C). The observed level of P-LRP6 in these cells were responsive to Wnt3a (3.4-fold increase) and inhibition by DKK1 (80% reduction in LNCaP cells), indicating functionality of a constitutively active Wnt–β-cat signaling pathway (Fig. 2D).

Figure 1. Cav-1 and LRP6 interaction in prostate cancer. A, positive association between Cav-1 and LRP6 expression in benign prostate epithelia (benign) adjacent to human primary prostate cancer, and bone metastases (Mets) was shown by immunohistochemical staining. Cav-1 and LRP6 levels were low in benign epithelia (top row), but Cav-1+ cancer cells exhibited strong LRP6 immunostaining (second row), whereas the Cav-1− prostate cancer cells showed minimal level of LRP6 (third row). In bone metastases, Cav-1+ cancer cells had high level of LRP6 (bottom). Original magnification, ×200. PCa, prostate cancer. B, the average LRP6 immunostaining score in the Cav-1+ prostate cancer was significantly higher than that in the Cav-1− area of the same prostate cancer tissue (mean ± SEM, *P < 0.05). C, PC-3 cells were treated with LRPsi, Cavsi, and NCsi and the expression of LRP6 and Cav-1 mRNA was determined by qRT-PCR analysis after 24 hours. Data are the mean ± SD relative to that in NCsi from 3 independent experiments. **P < 0.0001, (compared with NCsi). D, PC-3 cell lysates were prepared after 48 hours of transfection with LRP6 si, Cav-1si, or NCsi and analyzed by Western blotting. Cav-1− or LRP6-specific siRNA 1 and 2 are from Invitrogen and Santa Cruz, respectively.

Caveolin-1–LRP6 Signaling Stimulates Aerobic Glycolysis

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Although its conceivable that other Cav-1-mediated activities, for example, regulation of mRNAs, play a role, these data indicate that Cav-1 stimulates Wnt-β-caten signaling by increasing cellular levels of total and P-LRP6.

**Cav-1–LRP6 crosstalk stimulates aerobic glycolysis**

We investigated the role of Cav-1 and LRP6 in glycolytic metabolism in prostate cancer cells by analyzing the effects of Cav-1 and LRP6 knockdown in PC-3 cells and Cav-1 overexpression and LRP6 knockdown in LNCaP cells on the expression of selected proteins involved in glycolytic metabolism, including HK2 and Glut3. LRP6 knockdown in PC-3 cells markedly reduced protein levels of active β-caten, P-Akt (S473 and T308), Glut3, and HK2 (80%, 60%, 60%, 100%, and 80% respectively), and Cav-1 knockdown markedly reduced P-LRP6, active β-caten, and HK2 (80%, 60%, 80%, and 40%, respectively) and moderately reduced P-Akt and Glut3 levels compared with those in NCsi (Fig. 3A). These results suggest that Cav-1 and LRP6 increase glycolytic metabolism in prostate cancer cells, in part, by modulating Glut3 and HK2 expression.

We also examined the protein levels of other glycolytic enzymes including enolase 1 (ENO1), lactate dehydrogenase A, pyruvate kinase M2 (PKM2), and pyruvate dehydrogenase kinase 1 (PDK1), but the levels were unchanged in response to Cav-1 or LRP6 knockdown (data not shown). Further analysis showed that LRP6 and Cav-1 knockdown in PC-3 cells significantly reduced lactate production ($P < 0.01$, $P < 0.05$, respectively, vs. control NCsi, Fig. 3B), and that overexpression of Cav-1 by AdCa in LNCaP cells significantly increased lactate production ($P < 0.05$ vs. AdCt). In addition, LRP6si treatment...
significantly reduced lactate production in both AdCt- and AdCa-treated LNCaP cells (both $P < 0.01$, Fig. 3B). Furthermore, LRP6 and Cav-1 knockdown in PC-3 cells significantly reduced glucose uptake (both $P < 0.01$, Fig. 3B). Cav-1 overexpression in LNCaP cells significantly increased glucose uptake, whereas LRP6 knockdown significantly reduced it (both $P < 0.01$, Fig. 3B). These data indicate that both Cav-1 and LRP6 stimulate glycolytic activities in prostate cancer cells. Moreover, Cav-1 and LRP6 knockdown in PC-3 cells significantly reduce cell proliferation (both $P < 0.01$, Fig. 3D).

**Cav-1–LRP6 signaling stimulates aerobic glycolysis by activating Akt**

To determine the key downstream effector molecules in Cav-1–LRP6–stimulated glycolytic metabolism, we focused on the role of $\beta$-cat and Akt, examining the effects of $\beta$-cat knockdown and Akt inhibition on lactate production in PC-3 and LNCaP cells. Knockdown of $\beta$-cat in both cell lines did not significantly change lactate production relative to that of controls (Fig. 4A), indicating that Cav-1–LRP6 signaling enhanced glycolytic metabolism independently of $\beta$-cat signaling.

We next investigated the effect of Akt and its downstream effector mTORC1 on the glycolytic pathway by using an Akt inhibitor, Akt1/2, and an mTOR inhibitor, rapamycin, in PC-3 cells. Western blotting showed that both inhibitors reduced the expression of HK2 (70%), similarly to that observed in LRP6 and Cav-1 knockdown experiments. Of interest, both Akt1/2 and rapamycin also markedly reduced LRP6 phosphorylation (60% and 50% respectively, Fig. 4B). As expected, treatment of PC-3 with Akt1/2 markedly reduced P-Akt (S473) and P-S6K, and rapamycin treatment...
markedly reduced S6K phosphorylation compared with that of control but had no effect on Akt phosphorylation (Fig. 4B). Furthermore, treatment of PC-3 cells with Akti1/2 or rapamycin significantly reduced lactate production ($P < 0.01$, $P < 0.05$, respectively).

Cav-1 overexpression in LNCaP cells significantly increased lactate production, as expected ($P < 0.001$), and LRP6si, Akti1/2, and rapamycin treatments significantly reduced lactate production in both AdCt ($P < 0.05$, all comparisons) and AdCa ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) cells (Fig. 4C). Western blotting showed that Cav-1 and LRP6 knockdown markedly increased Raptor (S792) phosphorylation (2-, 1.7-fold, respectively) and total Raptor protein levels (2.7-, 2.3-fold, respectively), suggesting mTORC1 activity is stimulated through Raptor inhibition (Fig. 4D).

Overall, these results clearly suggest that Cav-1–LRP6 signaling stimulates glycolytic metabolism by activating Akt–mTORC1 signaling.

**Cav-1–LRP6 signaling activates Akt signaling by stimulating IGF-IR/IR phosphorylation**

Previous work showed a role for IGF-IR/IR signaling in regulating glycolytic pathways (45), so we considered whether Cav-1–LRP6 signaling might stimulate IGF-IR/IR phosphorylation, which in turn could activate Akt–mTORC1–mediated glycolytic metabolism.

We first examined the effect of Cav-1 and LRP6 on total and phosphorylated IGF-IR/IR expression. In PC-3 cells, Cav-1 knockdown reduced P-IGF-IR/IR relative to IGF-IR (50%) but only moderately reduced it relative to IR (10%), and LRP6
knockdown reduced P-IGF-IR/IR to undetectable levels and moderately reduced total protein levels of IR and IGF-IR. Cav-1 overexpression did not increase P-IGF-IR/IR levels in LNCaP cells but LRP6 knockdown reduced P-IGF-IR/IR 33% relative to IGF-IR, and 38% relative to IR in AdCa-treated cells, compared with that of NCsi (Fig. 5A).

To analyze the role of IR and IGF-IR in glycolytic metabolism, we further treated PC-3 cells with IR siRNA and IGF-IR siRNA, and stimulated LRP6 activity with Wnt3a. Wnt3a treatment increased P-LRP6, P-Akt (S473), and P-S6K (>10-, 1.6-, 9.2-fold, respectively) and increased P-IGF-IR/IR relative to total IR (1.4-fold) but did not change P-IGF-IR/IR relative to total IGF-IR. LRP6 and Cav-1 knockdown substantially reduced P-IGF-IR/IR relative to IGF-IR (100%, 70% respectively) and IR (100%, 71% respectively), P-Akt, (94%, 63%, respectively), and P-S6K (both 71%). Furthermore, IR and IGF-IR knockdown reduced Wnt3a-stimulated P-Akt (63%, 56%, respectively) and P-S6K (33%, 54%, respectively), but only IR knockdown reduced P-LRP6 by 27% (Fig. 5B).

We further examined the role of IR and IGF-IR on Wnt3a-stimulated P-LRP6, IR/IGF-IR, Akt, and S6K in PC-3 cells by using AG1204 (an IR and IGF-IR inhibitor) and picropodophyllin (PPP, an IGF-IR-specific inhibitor). AG1204 treatment did not change the P-LRP6 level, but PPP moderately (21%) reduced it. Furthermore, AG1204 treatment markedly reduced Wnt3a-stimulated P-IGF-IR/IR relative to total IGF-IR, total IR, P-Akt, and P-S6K (93%, 92%, 58%, 46%, respectively). Treatment with PPP, on the other hand, had no effect on Wnt3a-stimulated P-IGF-IR/IR relative to total IGF-IR but reduced it relative to total IR by 38%. PPP treatment also

![Figure 5](image-url)

**Figure 5.** LRP6 and Cav-1 stimulate IGF-IR/IR–mTORC1 signaling. A, PC-3 cells transfected with LRPsi, Cavsi, and NCsi. Cav-1 was overexpressed in LNCaP cells by infection with AdCa (or AdCt) and then transfected with LRPsi or NCsi. β-Actin was used as the loading control. B, PC-3 cells were transfected with LRPsi, Cavsi, IRsi, IGF-IRsi, and NCsi for 40 hours, were serum starved for 8 hours, and were treated with Wnt3a (50 ng/mL) for 30 minutes. C, PC-3 cells were serum starved for 8 hours, treated with Wnt3a (50 ng/mL) and PPP (0.5 μm/L) or AG1024 (1.0 μm/L) for 30 minutes. D, PC-3 cells were transfected as in B. LNCaP cells were infected with AdCa (or AdCt) and subsequently transfected with LRPsi, Cavsi, NCsi, IRsi, IGF-IRsi, or NCsi. The concentration of lactate accumulation in the medium of the treated PC-3 and LNCaP cells was measured after normalization to viable cells. Data are plotted as mean ± SD from 3 independent experiments. *P < 0.05, **P < 0.005. A–D, the cells were incubated for 48 hours and analyzed by Western blotting. E, LNCaP cells were transfected with Cav-1 and Cav-1 siRNA, then immunoprecipitated with anti-Flag, anti-5S mouse mAb, anti IR, or anti-IGF-IR rabbit pAb. Anti-Flag and anti-5S immunocomplexes were analyzed using rabbit pAb anti-Flag, anti-5S, anti-IR, and anti-IGF-IR, and the anti-IR or anti-IGF-IR immunocomplexes were analyzed using mouse mAb anti-Flag, anti-5S, anti-IR and rabbit mAb anti-IGF-IR.
reduced Wnt3a-stimulated P-Akt moderately (25%) but considerably reduced Wnt3a-stimulated P-S6K (85%). Of note, both AG1204 and PPP reduced Wnt3a-stimulated active β-cat (Fig. 5C). Similar crosstalk between insulin and Wnt–β-cat signaling pathways has been reported in intestinal endocrine L cells (44).

IR or IGF-IR knockdown significantly reduced lactate production in PC-3 cells relative to that of NCsi (P < 0.005, P < 0.05, respectively), similarly to that observed for Cav-1 and LRP6 knockdown (Fig. 5D). IR knockdown significantly reduced lactate production in both AdCt- and AdCa-treated LNCaP cells (P < 0.05, P < 0.005, respectively). IGF-IR knockdown significantly reduced lactate production only after Cav-1 overexpression (P < 0.05; Fig. 5D). Overall, these data indicate that IGF-IR/IR signaling activities are downstream of Cav-1–LRP6 signaling and induce Akt–mTORC1–mediated glycolytic metabolism.

We then examined whether Cav-1, LRP6, IR, and IGF-IR interact in prostate cancer cells. Cav-1 is known to interact independently with IR, IGF-IR, and LRP6 (12, 46, 47), but no available data point to the compartmentalized interaction and colocalization of these proteins. We overexpressed LRP6-Flag and Cav-1–V5-His in LNCaP cells and analyzed the communoprecipitation complexes produced from Flag-tag, V5-tag, IR, and IGF-IR immunoprecipitations. IR, IGF-IR, and Cav-1 were recovered in the LRP6-Flag communoprecipitation complexes, and those of Cav-1–V5 revealed IR and LRP6. LRP6 and Cav-1 were also recovered in both IR and IGF-IR communoprecipitation complexes (Fig. 5E).

Overall, our data suggest that Cav-1–LRP6–stimulated Akt–mTORC1–mediated glycolytic metabolism in prostate cancer cells can be initiated through the activities of a Cav-1–LRP6–IGF-IR/IR signaling module complex.

Discussion

Our results suggest a positive reciprocal mechanism promoting coordinate expression of Cav-1 and LRP6 in prostate cancer tissues and cells. We found that Cav-1 siRNA treatment substantially reduced cytoplasmic levels and nuclear translocation of active and total β-cat and reduced the accumulation of nuclear c-Myc levels in prostate cancer cells. We showed that Cav-1 knockdown reduced levels of total and P-LRP6 in prostate cancer cells that constitutively express canonical Wnt ligands (15–17). Together with the coreceptor FZD, LRP6 binds Wnt ligands and mediates canonical β-cat signaling in various cell types, including both normal and malignant epithelial and stromal cells (13, 14). Multiple Wnt genes or proteins (both canonical and noncanonical) are upregulated or mutated in primary prostate cancer, osteoblastic prostate cancer bone metastases, and CRPC (15–17). Previous work showed that Wnt3a can induce internalization of the FZD–LRP6 complex through Cav-1 (12). It has been shown that LRP6 contains 2 consensus Cav-1–binding motifs (Cav-1 scaffolding domain binding sites), 1 extracellular (F836–Y844) and 1 intracellular (Y1515–F1525), and that Cav-1 physically interacts with LRP6 (13). We now show that Cav-1, LRP6, IR, and IGF-IR interact with and bind to each other. Taken together, our results point to a Cav-1–LRP6 signaling module induced by the scaffolding functions of Cav-1, and driven by Wnt ligand stimulation in prostate cancer. Furthermore, these signaling module activities extend to crosstalk between Cav-1–LRP6 and IGF-IR signaling, leading to Akt activation and aerobic glycolysis, which provides new opportunities for development of new therapeutic concepts. However, it should be stressed that there is no consensus with regard to levels of expression and functional significance of specific elements of the IGF pathway in advanced prostate.

To our knowledge, LRP6-mediated stimulation of Akt and downstream activation of aerobic glycolysis in cancer has not been reported previously. A recent study showed that neurofilament-heavy polypeptide inhibits the Akt–β-cat pathway, reducing PKM2, increasing pyruvate dehydrogenase, and reducing aerobic glycolysis in human esophageal squamous cell carcinoma (48). Although our data showed that Cav-1–LRP6–mediated Akt activation led to increased aerobic glycolysis, we observed no change in the levels of PKM2, ENO1, LDHA, or PDK1 in PC-3 or LNCaP cells treated with Cav-1 and LRP6 siRNA. Our results also show that β-cat knockdown did not reduce lactate production in either PC-3 or LNCaP cells or cause any change in HK2 or Glut3 levels. These data suggest that Cav-1–LRP6–stimulated Akt phosphorylation and activation of aerobic glycolysis in PC-3 cells involves a different mechanism than the Akt–β-cat pathway previously reported for human esophageal squamous cell carcinoma (48). A recent report showing that Cav-1 stimulates aerobic glycolysis in colorectal cancer by stimulating HMGAl-mediated GLUT3 transcription (49) provides further support for our data. Our results also show that the Akt–mTORC1 pathway is responsible for upregulating HK2 and/or Glut3, as indicated by a marked increase in S6K phosphorylation following Cav-1 overexpression and by reduced phosphorylation following Cav-1 or LRP6 knockdown. Our studies further suggest a role for Raptor in regulating Cav-1–LRP6–mediated mTOR activities; however, additional studies are required to clarify our results.

Because previous reports have shown that PI3K signaling is the major cascade responsible for the metabolic actions of insulin (45), that Cav-1 is compartmentalized with and activates IGF-1R/IR signaling (21–23), and that evidence for crosstalk between Wnt and insulin signaling pathways exists (34–37), we investigated the mechanism by which Cav-1–LRP6 signaling activities lead to Akt–mTORC1 activation. We found that IGF-1R/IR phosphorylation was markedly reduced in PC-3 cells after LRP6 or Cav-1 knockdown. In addition, overexpression of Cav-1 in LNCaP cells increased IGF-1R/IR phosphorylation and that LRP6 knockdown abrogated this Cav-1–mediated IGF-1R/IR phosphorylation, suggesting that Cav-1 and LRP6 have an important role in insulin/IGF-I signaling, and placing LRP6 downstream of Cav-1 in this signaling cascade. These results, together with those in Fig. 3A, show that IGF-1R/IR stimulation is downstream of Cav-1–LRP6 signaling module activities. Although the role of Cav-1 in stimulating IGF-1R/IR phosphorylation is well documented (21, 47, 50), we found here a novel role for LRP6 in the stimulation of IGF-1R/IR–mediated glycolytic metabolism independent of β-cat signaling. These
effects could be achieved by a physical interaction between these molecules within lipid raft microdomains in prostate cancer cells.

Overall, we hypothesize that Cav-1 and LRP6 interact and promote their stability as well as stimulate LRP6 phosphorylation, which, through interaction with IGF-IR/IR in the lipid raft, leads to activation of insulin/IGF-1 signaling by increasing the phosphorylation of IGF-IR/IR. This increased phosphorylation of these receptors activates PI3K signaling, which results in increased phosphorylation of Akt and its downstream effector mTORC1. In turn, these effects result in increased expression and/or translocation of HK2 and Glut3, which directly stimulate aerobic glycolysis (Fig. 6). Further studies of this putative pathway are warranted on the basis of our findings.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Figure 6. Diagram illustrates that Cav-1–LRP6 crosstalk stimulates IGF-IR/IR phosphorylation in the lipid raft, causes activation of PI3K signaling, and results in increased expression of HK2 and Glut3, which directly stimulate aerobic glycolysis.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A. Tahir, G. Yang, A.A. Goltsov, C. Ren, J. Wang, W. Chang
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