

## CCL2 PROTECTS PROSTATE CANCER PC3 CELLS FROM AUTOPHAGIC DEATH VIA PI3K/AKT-DEPENDENT SURVIVIN UP-REGULATION

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Resistance to cell death is a hallmark of cancer. Autophagy is a survival mechanism activated in response to nutrient deprivation; however, excessive autophagy will ultimately induce cell death in a non-apoptotic manner. The present study demonstrates that CCL2 protects prostate cancer PC3 cells from autophagic death, allowing prolonged survival in serum-free conditions. Upon serum starvation, CCL2 induced survivin up-regulation in PC3, DU 145 and C4-2B prostate cancer cells. Both cell survival and survivin expression were stunted in CCL2-stimulated PC3 cells when treated either with the PI3K inhibitor, LY294002 (2 $\mu$ M) or the Akt-specific inhibitor-X, (Akti-X: 2.5  $\mu$ M). Furthermore, CCL2 significantly reduced LC3-II in serum-starved PC3; in contrast, treatment with LY294002 or Akti-X reversed the effect of CCL2 on LC3-II levels, suggesting that CCL2 signaling limits autophagy in these cells. Upon serum deprivation, the analysis of LC3 localization by immunofluorescence revealed a remarkable reduction in LC3 punctate after CCL2-stimulation. CCL2 treatment also resulted in a higher, sustained mTORC1 activity as measured by an increase in phospho-p70S6 kinase (Thr<sup>389</sup>). Rapamycin, an inducer of autophagy, both down-regulated survivin and decreased PC3 cell viability in serum-deprived conditions. Treatment with CCL2 however, allowed cells to partially resist rapamycin-induced death which correlated with survivin protein levels. In two stable transfectants expressing survivin-specific shRNA, generated from PC3, survivin protein levels controlled both cell viability and LC3 localization in response to CCL2 treatment. Altogether, these findings indicate that CCL2 protects prostate cancer PC3 cells from autophagic death via PI3K-Akt/survivin

pathway and reveal survivin as a critical molecule in this survival mechanism.

Extensive research has emerged in recent years revealing the roles of cytokines and chemokines, not only in their involvement in chemotaxis, but also in tumorigenesis and cancer cell proliferation, survival, adhesion and invasion (1-3). The CC class of chemokines, named for the first two conserved cysteine residues, have been shown to be highly involved in mechanisms of tumorigenesis within the skeletal systems, specifically: osteoclastic resorption, osteoblast induction, and bone remodeling (4). These cytokine mediated interactions in the bone microenvironment are critical to the promotion of cell survival and proliferation of metastatic tumors, which inevitably result in patient morbidity and mortality (5-7).

The CC chemokine, CCL2 (monocyte chemoattractant protein-1 (MCP-1)), while expressed only at low levels in prostate cancer cell lines, is highly secreted by human bone marrow endothelial (HBME) cells. It is found in the microenvironment of prostate cancer bone metastases, where it has multiple effects on both tumor and host cells (7). CCL2 dose-dependently induces prostate cancer cell proliferation and invasion (8) and regulates monocyte and macrophage infiltration to prostate cancer epithelial cells, promoting tumorigenesis (9).

Chemokine-mediated signal transduction is initiated through binding to seven-transmembrane-domain heterotrimeric G-protein coupled receptors (GPCRs) (10,11). Specifically, CCL2 interacts with the CCR2B integral membrane receptor, which is responsive to all members of the CC chemokine family (10,12). The current proposed mechanism suggests that CCL2 stimulation activates a signaling cascade, in which the G $\beta\gamma$  subunits dissociate from the

heterotrimeric  $G\alpha\beta\gamma$ -receptor complex. Following dissociation, the  $G\beta\gamma$  heterodimer will either rapidly activate, by direct interaction, two phosphoinositide-specific lipase isoenzymes,  $PLC\beta_2$  and  $PLC\beta_3$ , or  $PI3K\gamma$ , resulting in downstream activation of PKC and PKB (Akt) or the mitogen activated protein kinase (MAPK) cascade, respectively (13). In addition, G-protein complex dissociation induces activation of PI3K via the  $G\alpha$  subunit, which, in turn, either activates PKB/Akt via phosphorylation at Thr<sup>308</sup> and Ser<sup>473</sup> by PDK1 and PDK2, respectively, or the MAPK cascade via intermediate cytoskeleton-associated kinases (10,13,14). Activation of PKB/Akt thereafter leads to a wide array of downstream functional biological responses which may prolong cell survival, including, but not limited to, induction of endogenous survivin expression (15,16).

Macroautophagy (referred to as autophagy herein) is a highly evolutionarily conserved catabolic mechanism responsible for the removal and breakdown of cellular materials as a means to maintain intracellular homeostasis (17,18). Functioning at normal basal levels, autophagy is one of the major mechanisms for the degradation of proteins, macromolecules, damaged organelles, and other unwanted structures (19). However, autophagosome formation can also be stimulated as a response to stresses, such as oxidative damage and nutrient deprivation, functioning to remove protein aggregates and provide required amino acids essential for metabolic processes and cell survival (17,18). Excessive autophagy will also inevitably trigger autophagic cell death or "type II programmed cell death" (type I programmed cell death = apoptosis). Due to emerging links between autophagic aberrations and cancer, there is rapidly growing support that this type II programmed cell death may be also function as a suppressor of tumorigenesis (18).

Autophagic degradation of intracellular materials is initiated by the formation of double-membrane bound vacuoles, termed autophagosomes, which consume part of the cell's cytoplasm, including elements to be degraded. Digestion is completed upon autophagosome maturation, via fusion with endocytic lysosomes. This process requires activation of the downstream product of class-III PI3K, phosphatidylinositol 3-phosphate. Conversely, products of class-I PI3K,

phosphatidylinositol-3,4-biphosphate and phosphatidylinositol-3,4,5-triphosphate exhibit an inhibitory effect (17). When nutrients are abundant, class-I PI3K promotes activation of the Ser/Thr protein kinase, mammalian target of rapamycin (mTOR) through RTKs, mTOR thereby suppresses autophagosome formation (18). On the other hand, LY294002-mediated inhibition of PI3K and negative regulation of mTOR by rapamycin both significantly induce autophagy. mTOR activates downstream Ser/Thr kinase, P70S6, which thereafter phosphorylates ribosomal protein S6 (17-19) which is reliably detectable by immunoblotting and serves an accurate measure of cellular autophagy for this study. During Autophagosome formation, cytosolic microtubule-associated protein light chain 3-I (LC3-I) is conjugated with phosphatidylethanolamine (PE) and converted to LC3-II. This PE-conjugated LC3-II, detectable by immunoblotting, is present specifically on isolation membranes and autophagosomes (20), and therefore serves a second and widely accepted approach to monitoring autophagic flux for this study.

Although CCL2 is widely accepted to play a direct role in inflammation, tumorigenesis, and chemotaxis, its role in cell survival has not been extensively examined. In the present study, we demonstrate that CCL2 confers a distinct survival advantage to PC3 prostate cancer cells by regulation of autophagic death. Furthermore, we have established that CCL2 functions primarily through a PI3K-Akt dependent pathway following up-regulation of the survival gene, survivin (BIRC5).

## EXPERIMENTAL PROCEDURES

*Cell Lines-* The androgen-independent human prostate cancer cell lines, PC3 and DU 145 were obtained from ATCC (Manassas, VA). Cells were maintained in RPMI 1640 (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% Antibiotic-Antimycotic (Invitrogen). The human prostate C4-2B cells (UroCor, Inc., Oklahoma City, OK) were derived from LNCaP cells through several passages via castrated nude mice and isolated from the tumor that metastasize to bone (21). The C4-2B cells were maintained in T medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and

1% Antibiotic-Antimycotic (Invitrogen). Regular passaging was performed at 70-80% confluency by trypsinization, using 1x trypsin and 0.05% EDTA, followed by resuspension in complete media. Cells were grown and maintained at 37°C and 5% CO<sub>2</sub>, 95% air atmosphere.

*WST-1 Cell Viability Assay*- Dye conversion at 440nm of 4-[3-(4-Idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate to formazan (Cell Proliferation Reagent WST-1; Roche) was used to assess cell viability and chemosensitivity of PC3 cells. Cells were grown to 80% confluency then serum-starved for 16-18 hours. Synchronized cells were plated at 10<sup>4</sup> cells per well into 96-well flat-bottom tissue culture plates (Costar; #3596) and were allowed to attach for 6 hours. Cells were then treated with increasing concentrations of inhibitors and/or CCL2 chemokine (Apollo Cytokine Research, Australia) or IGF-1 (R&D Systems, #291-G1) and incubated for 24-168 hours. WST-1 reagent was added following manufactures instructions and plates were returned to 37°C for 105 minutes. Dye conversion at an absorbance of 440nm was ascertained by the VERSAmax Microplate Reader and analyzed using Softmax Pro 3.12 software. The aforementioned technique was applied to dose-response and time-course assays to evaluate the effects of the following inhibitors: LY294002 phosphoinositide-3-kinase inhibitor (Cayman Chemical, MI; 709920), Akt inhibitor X (Akti-X, Calbiochem; 124020) (22). Rapamycin (Calbiochem; 553211) was also assessed using this technique; however, prior to treatment, PC3 cells were incubated for 24 hours and the cell viability was evaluated 24 hours post-treatment.

*LIVE/DEAD<sup>®</sup> Cell Viability Assay*- PC3 cells were serum-starved in RPMI 1640 media for 16-18 hours, then plated into Lab-Tek II dual chamber slides (Nalge Nunc International; #155379) at a density of 2.5 x 10<sup>5</sup> cells/ml. Cells were allowed to attach for 6 hours and CCL2 (100 ng/ml) was added to one chamber of each slide. Cells were then incubated for 24-168 hours and viability of CCL2 treated and untreated PC3 cells was evaluated using the LIVE/DEAD<sup>®</sup> Viability/Cytotoxicity Kit \*for mammalian cells\* (Invitrogen), according to manufactures instructions. Immunofluorescence was visualized using an Olympus IX71 microscope. Images were captured using a 20x objective, at 560nm and

535nm excitation wavelengths and 645nm and 610nm emission wavelengths, respective to each reagent dye.

*Real-Time PCR*- cDNA was generated from control and CCL2 treated PC3 cells at 72 hours using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) according to manufactures instructions. Real-time PCR analysis was performed using Taqman Gene Expression Master Mix (Applied Biosystems, #4369016) and *Survivin*-specific Taqman Pre-Designed Probe Assay (Applied Biosystems, #Hs00977611\_g1). Finally, mRNA expression analysis was completed by the University of Michigan Microarray Core Facility, using the ABI 7900 HT sequence detection system.

*Western Blot Analysis*- Prostate cancer cells (PC3, DU 145, C4-2B) were grown to 80% confluency on 150 cm<sup>2</sup> tissue culture flasks (Falcon; NJ) in appropriate media, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. FBS-containing media was removed and flasks were washed 4 times with HANKS buffered medium (Invitrogen). PC3 and DU145 cells were synchronized by starvation in serum-free RPMI 1640 for 16-18 hours at 37°C. Cells were detached with mild treatment, using 0.25 mM EDTA pH 8.0 (Invitrogen), at 37°C for 20-25 minutes, and then plated in either 6-well or 100mm culture plates at a density of 2.5x10<sup>5</sup> cells/ml. Cells were allowed to attach for 6 hours prior to treatment with inhibitors (as specified above) and/or CCL2/IGF-1 (100 ng/ml). C4-2B cells were plated in complete medium and 16 hours later the medium was replaced by serum free T-medium containing CCL2 (100 ng/ml). After treatment, cells were harvested at increasing time points in RIPA cell lysis buffer (150mM NaCl; 50mM Tris base, pH 8.0; 1% NP-40; 0.5% DOC ; 20% SDS; 1μM okadaic acid; 1μg/ml aprontinin, leupeptin, and pepstatin). Protein samples were sonicated, followed by centrifugation at 13,000 rpm for 10 minutes. Supernatants were collected and protein concentrations were determined using the Bradford Assay (Bio-Rad). Protein lysates were electrophoresed through 4-20% Tris-Glycine SDS polyacrylamide gel electrophoresis and transferred onto PVDF according to Invitrogen instructions. Membranes were blocked in 5% milk-TBST (Tris-buffered saline; 0.1% Tween) for 1 hour at room temperature, then incubated

overnight at 4°C with primary (rabbit) antibodies (Cell Signaling), diluted 1:1000 in blocking solution. 16-24 hours later, membranes were washed 3 times with TBST and incubated for 1 hour with goat anti-rabbit IgG, HRP-Conjugated antibody (Cell Signaling; #7074), diluted 1:2000 in blocking solution. Protein expression was visualized by ECL chemiluminescence (Millipore). Finally,  $\beta$ -Actin monoclonal antibody (Cell Signaling; #4967L) was used as a loading control for all membranes.

**Cell Cycle Analysis-** PC3 cells were serum-starved in RPMI 1640 for 16 hours and then plated ( $2 \times 10^6$  cells/plate) into 100mm tissue culture plates. Cells were allowed to attach for 6 hours. One half of the plates were then treated with CCL2 (100 ng/ml) and cells from both treated and untreated groups were collected at 48, 72, 96, and 120 hours, following standard trypsinization procedure. Collected cells were resuspended in 500 $\mu$ l PBS (Invitrogen) and fixed by adding 500 $\mu$ l 100% EtOH (equilibrated to -20°C). Fixed cells were stored for a minimum of 20 minutes at 4°C until samples from all aforementioned time points were collected. Following fixation, cells were pelleted, EtOH/PBS was decanted, and cells were resuspended in 500 $\mu$ l PI-RNase solution (50  $\mu$ g/ml Propidium Iodide (Invitrogen), 100  $\mu$ g/ml RNase A (Qiagen), diluted in PBS), then incubated at room temperature for 20 minutes. Propidium iodide (PI) stained samples were analyzed on a FACSCalibur (Becton Dickinson) with Cell Quest Pro (Becton Dickinson) software. Viable cells were gated based upon forward scatter (FSC) and side scatter (SSC) parameters. PI fluorescence was detected in the FL2 channel on a logarithmic scale and in the FL3 channel on a linear scale. Voltage adjustments were performed for each sample to place the  $G_0/G_1$  peak at a standard of 400 on the linear scale. For each sample,  $10^4$  events were collected for analysis as the Boolean intersection of viable cells, based upon FSC/SSC gate. Single cell analysis was based upon FL3 width v. FL3 area doublet discrimination gate. The relative percents of cells found within each phase of the cell cycle were determined with ModFit LT cell cycle analysis software (Verity House). A linearity factor ( $G_2/G_1$ ) of 1.84 was used for all samples. Sub  $G_0/G_1$  DNA content (apoptotic cells) was assessed by analysis of PI fluorescence detected in the FL2 channel.

**Transient Transfections-** PC3 cells were synchronized in 150 cm<sup>2</sup> tissue culture flasks, as previously described, then plated in serum-free RPMI 1640 at  $10^4$  cells/well, in 96-well plates. Plates were incubated at 37°C, 5% CO<sub>2</sub> overnight. The following day, cells were transfected with either *Survivin*-specific or non-silencing siRNA at both 50 and 100 nM, according to instructions provided with the SignalSilence (R) survivin siRNA Kit (Cell Signaling; 6350S). Cells were transfected in replicates of 10 for each concentration of siRNA. Following transfection, plates were incubated for an additional 24 hours. CCL2 (100 ng/ml) was then added and the cells were further incubated for 72 hours. Finally, cell viability was assessed by WST-1 dye conversion, as previously described.

**Generation of Stable Cell Lines-** Two independent *survivin* shRNAs (shS-1 and shS-2; Open Biosystems: RHS4430-98520325, RHS4430-99140887, respectively) were packaged into lentivirus by the University of Michigan Vector Core, using psPAX2 and pMD2.G mammalian expression lentiviral helper plasmids (Addgene; 12260; 12259). PC3 cells were trypsinized and seeded at  $10^6$  cells per 100mm tissue culture plate in RPMI 1640 supplemented with 10% FBS and 1% antibiotic-antimycotic. Cells were allowed to grow in complete medium for 24 hours. Following incubation, media was replaced with 10ml per plate of fresh FBS-containing RPMI 1640. To transfect PC3, 200 $\mu$ l of each lentiviral shRNA (shS-1 and shS-2, 10x concentrated) was added drop-wise to previously seeded cells, in triplicate. Plates were transferred immediately to 37°C, 5% CO<sub>2</sub> for 24 hours. Cells were then trypsinized and plated in complete media, containing 5 $\mu$ g/ml Puromycin (Sigma; P9620-10ML) and incubated for 48 hours. Following incubation, cells were washed three times with HANKS balanced salt solution to remove unattached cells, and medium was replaced with 10ml RPMI 1640, containing 2 $\mu$ g/ml Puromycin. Cells were supplemented with this media every 3-4 days, until GFP-expressing colonies were observed. GFP immunofluorescence was assessed using the Olympus IX71 microscope (20 x magnification) fitted with a 560nm excitation and 645nm emission filter. Visible colonies were trypsinized and plated into 100 mm tissue culture plates, then grown to 80% confluency in the



presence of 2 $\mu$ g/ml Puromycin prior to cell viability and Western blot analysis, as described above. Stable cell lines were named shS-1 and shS-2, corresponding to transfected *survivin* shRNA.

**Immunofluorescence** – PC3, shS-1, and shS-2 cells were serum starved in RPMI 1640 media for 16 hours at 37°C, then plated into Lab-Tek II dual-chamber culture slides (Nunc, #177380) and allowed to attach at 37°C for 6 hours. Cells were then either left untreated or treated with CCL2 (100 ng/mL) and incubated for 72 hours at 37°C. Cells were washed briefly with PBS and then fixed with 3% formaldehyde-PBS for 15 minutes. Fixed cells were then incubated with LC3B primary antibody (diluted 1:200 in PBS/Triton) for 24 hours, as per Cell Signaling recommended procedures. Following incubation, cells were washed as per manufacturer's instructions and then incubated with fluorochrome secondary antibodies (diluted 1:200 in PBS/Triton) for 2 hours at room temperature, in dark. For parental PC3 cells AlexaFluor 488-conjugated goat-anti-rabbit IgG (Invitrogen, #A11034) was used as secondary antibody, whereas for shS-1 and shS-2 cells the secondary antibody was replaced by AlexaFluor 647-conjugated goat-anti-rabbit IgG (Invitrogen, #A21245) to prevent fluorescence interference with the GFP incorporated during stable transfection. Following secondary staining, coverslips were applied with Prolong<sup>®</sup> Gold Antifade reagent (Invitrogen, #P-36930), and slides were transferred to 4°C for at least 24 hours prior to immunofluorescence analysis. Images were obtained using an Olympus IX-71 FluoView 500 Laser Scanning Confocal Microscope at 60X magnification with a PlanApo N oil immersion lens, with a numerical aperture of 1.42. Flow View TIEMPO software version 4.3 was used to obtain all Z-stack images and the corresponding maximum intensity projections.

**Immunoprecipitation** – PC3 cells were grown to 80% confluency and then synchronized for 16 hours by serum starvation. 3.5 $\times$ 10<sup>5</sup> cells were plated into 100mm tissue culture plates (Sarstedt, #83.1802) and allowed to attach for 6 hours at 37°C. Following attachment, plates were treated or not with CCL2 (100 ng/mL) for 48 hours at 37°C. Cells  $\pm$  CCL2 were harvested in 300 $\mu$ l/plate cell lysis buffer (Cell Signaling, #9803), containing 1 $\mu$ M okadaic acid. Cell extracts were

obtained by sonication and centrifugation for 20 minutes at 13000 rpm. 50 $\mu$ l of cell lysate from each condition (+/- CCL2) was removed and stored at -20°C for the analysis of inputs. Samples were precleared for 2 hours at 4°C with 150 $\mu$ l, per condition, of Dynabeads Protein G (Invitrogen, #100.04D). After preclearing, survivin rabbit mAb (Cell Signaling, #2808) or normal rabbit IgG (Cell Signaling, #2729S) antibodies were added to cell lysates at 1:100 or 1:200 dilutions and incubated at 4°C overnight. Dynabeads were washed with cell lysis buffer and placed onto Dynal MPC<sup>™</sup> magnets (Invitrogen, #120.20D) for 2 minutes to remove wash. Samples were added to washed beads and the immunoprecipitation was carried out following Invitrogen recommended instructions, however all washes were performed using cell lysis buffer (Cell Signaling). Immunoprecipitates and inputs were separated by electrophoresis on a 4-20% SDS-PAGE, and then transferred to PVDF membranes following standard western blotting procedure. Membranes were incubated overnight with LC3B antibody (Cell Signaling, #2775), diluted 1:1000 in 5% milk-TBST, followed by HRP-conjugated secondary antibody (Cell Signaling, #7074). Membranes were also probed with survivin mAb (Cell Signaling) to verify efficiency and specificity.

**Statistical Analysis**- All average values are presented as means  $\pm$  SE (standard errors). Data were analyzed using GraphPad Prism software and one-way ANOVA combined with Bonferroni's test. A probability level of less than 0.05 was considered significant.

## RESULTS

*CCL2 signaling protects PC3 cells from death upon serum deprivation.* The identification of CCL2-mediated up-regulation in the tumor-bone microenvironment of prostate cancer metastases (7) requires a greater understanding of the role this chemokine plays in the development and progression of prostate cancer. The studies here reveal that CCL2 is a powerful inducer of PC3 cell survival upon serum deprivation. Survival was analyzed at different times by WST-1 assay. At concentrations of 50 and 100 ng/mL, CCL2 maintained cell viability up to 168 hours of serum starvation (Fig. 1A). This result was further

corroborated using the Live/Dead<sup>®</sup> Viability/Cytotoxicity Assay which allows simultaneous determination of live (green) and dead (red) cells. CCL2 protection from cell death was evidenced by a higher average ratio (3 to 5 times) of live:dead cells as compared to control cells (Figs. 1B and 1C). After two weeks, the majority of cells exposed to CCL2 were able to survive in contrast to control cells, suggesting that CCL2 confers an advantage upon PC3 cells to survive in adverse conditions such as limited nutrients and growth factors (Fig. 1D). Furthermore, after four weeks in serum-free media, CCL2-stimulated cells responded to re-addition of complete medium (10% FBS) by a rapid restoration of the proliferative potential and recovery of the original size and morphology, while the control cells were unable to survive (Fig. 1E).

To determine whether prolonged cell survival upon serum deprivation is CCL2 signaling-specific, PC3 survival was assessed by comparison to another factor known to induce Akt activation, IGF-1. The viability of the cells treated with IGF-1 was no different than the untreated cells (Fig. 1F), demonstrating that in contrast to CCL2, IGF-1 was unable to induce cell survival. These results also suggest that CCL2 induces specific signals that protect PC3 from cell death upon serum deprivation.

*CCL2-mediated cell survival is PI3K-Akt pathway-dependent.* To determine the mechanism involved in CCL2-induced cell survival, we investigated the signaling pathways activated by this chemokine that are critical for cell survival. Previous data have shown that CCL2 induces Akt phosphorylation in prostate cancer cells, a known regulator of cell survival and proliferation, in response to intracellular and extracellular stimuli (23, Loberg, 2006 #4). Full activation of Akt requires the phosphorylation of two key regulatory sites: Ser<sup>473</sup> and Thr<sup>308</sup>. CCL2 induces a rapid (15 min post-stimulation) increase in Akt phosphorylation both at Ser<sup>473</sup> and Thr<sup>308</sup> residues (Fig. 2Aa,b). Because of a PTEN mutation, these cells exhibit a constitutive Akt phosphorylation; however, our results indicate that a hyper-phosphorylation and activation of this molecule takes place upon CCL2 signaling.

The significance of PI3K-Akt pathway for cell survival was evaluated in a series of time-

course experiments by measuring the cell viability in serum-free media using either LY294002, a PI3K-specific inhibitor (24), or Akti-X, a selective inhibitor of Akt phosphorylation with no effect on PI3K or PDK1 (22). Fig. 2Ba shows the survival curves for control and CCL2-stimulated cells (100ng/ml) in the presence of LY294002 using two different concentrations of the inhibitor: 1 and 2  $\mu$ M. As observed in this figure, LY294002 at 1  $\mu$ M caused a significant decrease in the viability of CCL2-treated cells, and at 2  $\mu$ M it completely abrogated the survival induced by this chemokine. However, when the inhibitor was used at this concentration (2  $\mu$ M) in control cells, it did not cause any further decrease in cell viability. Similar results were obtained with the specific Akt inhibitor: Akti-X (Fig. 2Ca). When the inhibitor was used at 1.25  $\mu$ M (IC<sub>50</sub>=2-5  $\mu$ M, Calbiochem) a decrease in cell viability was observed and a complete abrogation of CCL2-induced survival was obtained in the presence of Akti-X at 2.5  $\mu$ M. The inhibition of Akt phosphorylation was confirmed by immunoblotting analysis of cell lysates corresponding to CCL2 treated cells in response to LY294002 (2  $\mu$ M) or Akti-X (1.25, 2.5 and 5  $\mu$ M). To detect Akt activation, cell extracts were probed with phospho-Akt specific antibodies (Ser<sup>473</sup> and Thr<sup>308</sup>). The results shown in Figs. 2Bb and 2Cb revealed that both LY294002 and Akti-X effectively inhibited the Akt phosphorylation at both residues. Altogether, these results demonstrate that the activation of the PI3K-Akt pathway is essential in the cell survival mechanism induced by CCL2.

*CCL2 signaling increases survivin expression via a PI3K-Akt dependent mechanism.* The effect of CCL2 on the expression of the inhibitors of apoptosis (IAP) family proteins was examined by Western blotting. Control and CCL2-stimulated PC3 cells were maintained in serum-free media up to 96 hours and the cell extracts were collected at increasing time points for analysis with different IAP antibodies: survivin, cIAP1, cIAP2, XIAP, Bcl2, Bcl-XL. In non-stimulated control cells, the survivin protein levels were dramatically down-regulated after 24 hours in serum-free conditions (Fig. 3A). In contrast, survivin was highly up-regulated in CCL2-treated cells when compared with control after 24 hours. On the other hand, none of the other analyzed proteins showed a significant change as a result of

CCL2 treatment. The anti-apoptotic proteins c-IAP1, Bcl2 and Bcl-XL exhibited some up-regulation in time, but no significant differences were observed between control and CCL2 samples.

The transcriptional regulation of *Survivin* in response to CCL2 signaling was also explored by Real-time PCR. Control and CCL2-treated cells were maintained 72 hours in serum-free media prior to mRNA analysis. As shown in Fig. 3B, the Real-time results from three independent samples of each control and CCL2-stimulated cells evidenced a greater than three-fold increase in Survivin mRNA as a result of CCL2 treatment, suggesting that survivin regulation occurs, to some degree, at the transcriptional level. At this time point, significant up-regulation was also observed in survivin protein (box in Fig. 3B).

Because CCL2-induced survival is PI3K-Akt dependent, it was reasoned that if survivin were an essential factor in this mechanism, then its up-regulation would also be PI3K-Akt dependent. To test this hypothesis, CCL2-stimulated cells were treated with or without LY294002 (2  $\mu$ M) or Akti-X (1.25, 2.5 and 5  $\mu$ M) and cell lysates were collected for immunoblotting at different times. As shown in Fig. 3C, LY294002 abrogated the CCL2-mediated up-regulation of survivin, resulting in a pattern similar to non-CCL2-stimulated samples as previously observed (see Fig. 3A). Similarly, the specific Akt inhibitor (Akti-X) evidenced a dose-dependent decrease of survivin protein levels in CCL2-stimulated cells. The survivin down-regulation induced by Akti-X was first observed at 24 hours and became more evident at 48 and 72 hours (Figs. 3Da,b). Thus, survivin up-regulation by CCL2 signaling correlates well with cell survival and is also PI3K-Akt dependent.

The specificity of CCL2-dependent survivin up-regulation was further assessed by immunoblot analysis of protein lysates from PC3 cells stimulated with IGF-1. Although IGF-1 effectively induced the Akt hyper-phosphorylation in these cells, this signaling did not induce survivin up-regulation (Fig. 3E). As IGF-1 was also unable to induce cell survival (Fig. 1F) these results support the strong correlation between cell survival and survivin expression and further demonstrate the specificity of CCL2 signaling. Furthermore, because IGF-1 activates Akt and the survivin up-regulation induced by CCL2 is PI3K-

Akt dependent, these findings suggest that other signaling molecules may interact or synergize with the Akt signaling pathway to produce the up-regulation of survivin.

Several studies have shown that survivin expression inhibits cell death induced by various apoptotic stimuli *in vitro* (15,25,26) and *in vivo* (16,27,28). This strongly suggests that survivin could be a critical factor in the mechanism of CCL2-mediated survival.

*CCL2 signaling induces survivin up-regulation in other prostate cancer cells.* The effect of CCL2 in survivin regulation was also investigated in C4-2B and DU 145 prostate cancer cells upon serum deprivation. Cell-lysates from control and CCL2-stimulated cells were collected at different times for the analysis of survivin protein expression by Western blot. Similar to PC3, upon serum withdrawal, survivin protein levels were dramatically down-regulated in non-stimulated C4-2B and DU 145 cells. However, survivin protein was significantly up-regulated in CCL2-treated cells when compared to control, as observed after 24 hours in serum free media (Figs. 4a,b). These results suggest that an analogous mechanism could be activated in other prostate cancer cells.

*Autophagy is activated in PC3 cells upon serum deprivation and CCL2 protects cells from autophagic death.* Subsequent analysis was focused on identification of the mechanisms of PC3 cell death and CCL2 protection upon serum withdrawal. Apoptosis was assessed at different times (48 to 120 hours) by propidium iodide staining of control and CCL2-treated cells based on the analysis of sub-G<sub>1</sub> cell population by Flow Cytometry. The results evidenced very low apoptotic (hypodiploid) cell populations (less than 1%) and no significant differences between control and CCL2-stimulated cells (Fig. 5). Furthermore, a cell cycle analysis of these populations indicated that the majority of cells were arrested in G<sub>1</sub> (about 80%) for both control and CCL2-cells. Additionally, activated caspases were not identified by immunoblot analysis (not shown). Because cellular death was evident in control cells and suppressed by CCL2 (see Fig. 1A-E), these results imply that the mechanism of death is actually non-apoptotic and caspase-independent. These data correlate with previous reports suggesting that the high level of endogenous Bcl-

XL expression protects PC3 cells from apoptotic signals in serum-free media (29).

Autophagy, a mechanism activated in response to starvation (30), has the capacity to cause cell death in a non-apoptotic manner and is considered the second most common form of programmed cell death. Microtubule-associated protein light chain 3 (LC3) is widely used to monitor autophagy (20). During autophagy, LC3-I is cleaved and conjugated to phosphatidylethanolamine to form LC3-II and this processing is essential for the formation of the autophagosome (31). Since the amount of LC3-II correlates with the number of autophagosomes, it serves as a good indicator of autophagosome formation (31); consequentially, the analysis of autophagy in response to CCL2 was based on LC3 immunodetection. However, because LC3-II itself is degraded by autophagy, the cells were also treated with protease inhibitors (E64d and Leupeptin) to detect autophagic flux (20). The results shown in Fig. 6A demonstrated that autophagy was induced both in control and CCL2-stimulated cells: First, the amount of LC3-II was lower in the FBS sample (low autophagy) and increased in time under serum deprivation, suggesting an increase in the number of autophagosomes. Second, a further increase in LC3-II was observed in response to treatment with protease inhibitors (E64d and Leupeptin), which served as an indicator of autophagic flux. To determine the possible effect of CCL2 on the extent of autophagosome formation, immunoblot analysis and quantification of LC3-II bands was performed with lysates collected at 24 and 72 hours post-CCL2 treatment (Fig. 6Ba,b). The quantification analysis revealed a significantly lower amount of LC3-II in cells treated with CCL2: more than 4 fold at 24 hours and over 2 fold at 72 hours. Because the amount of LC3-II correlates with the number of autophagosomes, these results strongly suggest that CCL2 limits the autophagosome formation and consequently, the overall autophagy in these cells. In addition, Western blot analysis was performed to compare LC3-II protein levels of CCL2-cells in response to LY294002 treatment. The extracts from LY294002-treated cells exhibited a gradual increase in LC3-II when compared to untreated cells (Fig. 6Ca,b). At 96 hours LC3-II protein was two fold higher in CCL2+LY294002 samples than

in CCL2 alone. Similarly, the Akt-specific inhibitor (Akti-X) induced a dose-dependent increase of LC3-II in CCL2-stimulated cells (Fig. 6D). These findings indicate that CCL2 controls autophagosome formation via a PI3K/Akt-dependent pathway, which correlates with CCL2-mediated cell survival and protection from excessive autophagy as a PI3K/Akt-dependent mechanism.

Upon induction of autophagy changes in the localization of LC3 occur as a result of its recruitment to autophagic membranes and these changes are observed as an increase in punctate LC3 (31,32). LC3 localization in control and CCL2-treated cells was evaluated 72 hours post-stimulation by immunofluorescence microscopy using the LC3B antibody. As shown in Fig. 6E control cells (a) evidenced a remarkable increase in LC3 punctae (representing the LC3-II colocalization) as compared with the CCL2-treated cells (b) which exhibited more uniform distribution of the signal as well as lower fluorescence intensity. This result correlates the lower amount of LC3-II detected by Western blot in CCL2-stimulated cells with reduced autophagosome formation.

To further support the hypothesis that CCL2 protects PC3 cells from autophagic death, cells were treated with rapamycin, an inhibitor of mTORC1 (mTOR complex 1 or raptor-mTOR complex) and inducer of autophagy (17,33-36). In this experiment, cells were plated in serum free media for 24 hours and then treated as indicated with CCL2 and increasing rapamycin concentrations. The analysis of cell viability 24 hours post-treatment is shown in Fig. 7Aa,b. Fig. 7Aa illustrates WST-1 values for control and CCL2 cells in response to rapamycin. In Fig. 7Ab, the results presented are normalized to 0 nM rapamycin for each condition ( $\pm$  CCL2) and expressed as percent survival. From these results, it can be ascertained that rapamycin increases PC3 cell death and CCL2 provides partial protection from this effect. To investigate a possible correlation between rapamycin-induced cell death and survivin protein levels, a parallel Western blot analysis of these samples was performed as indicated in Fig. 7B. In CCL2 treated cells, it was evident that rapamycin partially abrogated survivin up-regulation (Fig. 7B, lanes 3 & 4); however, in control cells, rapamycin-induced



survivin down-regulation was masked due to the low protein expression observed after 48 hours in the untreated PC3 (Fig. 7B, lanes 1 & 2). In summary, these findings suggest that survivin plays a critical role in CCL2-mediated protection from autophagic death.

On the other hand, because mTORC1 negatively regulates autophagy and directly phosphorylates the p70S6 kinase on Thr<sup>389</sup> (a residue whose phosphorylation is rapamycin-sensitive *in vivo* and necessary for S6 kinase activity) (37), it has been proposed that mTORC1 activity (and so autophagy) can be monitored by following the phosphorylation of its target protein: p70S6K (32). Consequently, Western blot analysis was performed with lysates from control and CCL2-treated PC3 cells (+/- Akti-X) to detect the p70S6K phosphorylation. As shown in Fig. 7C, CCL2 induced higher levels of phospho-p70S6K at both 48 and 72 hours post-treatment. The higher phosphorylation of p70S6K correlated with lower LC3-II (as indication of lower autophagic activity) and higher expression of survivin. Furthermore, the specific Akt inhibitor (Akti-X) induced a dose-dependent decrease of phospho-p70S6K in CCL2-treated cells, correlating with higher LC3-II and lower survivin. In contrast, while IGF-1 was able to induce early (0.25-0.5 hours) phosphorylation of p70S6K, stimulation completely disappeared by 24 hours (Fig. 7D). These results are consistent with the inability of IGF-1 to protect PC3 from serum-deprivation stress (see Fig. 1F).

*Survivin is an essential factor for CCL2-mediated survival.* The role of survivin in the CCL2-induced survival mechanism was further investigated by transient knockdown experiments using *Survivin*-specific siRNA. PC3 cells were transfected with two different concentrations (50 and 100 nM) of either *Survivin*-specific or non-silencing siRNA. The cell viability was then analyzed in response to CCL2 stimulation by WST-1 dye conversion. It can be inferred from Fig. 8A, that in CCL2-stimulated cells *Survivin* siRNA substantially reduces cell viability as compared to control siRNA (non-silencing). On the other hand, no major differences were observed in non-CCL2 stimulated cells. Immunoblot analysis of lysates from cells transfected with control and *Survivin* siRNA demonstrated the effectiveness of *Survivin* siRNA

to reduce the survivin protein expression (Fig. 8B).

Additionally, two stable cell lines (shS-1 and shS-2) expressing short hairpin RNA (shRNA) targeting survivin were generated from PC3. WST-1 assay was performed to compare the PC3 control with shS-1 and shS-2 cell lines, in response to CCL2 stimulation. Immunoblot analysis of cell extracts from both shS-1 and shS-2 evidenced a reduction in survivin expression, compared to the parental control cells (Figs. 9D and 9H). However, some differences were observed between these cell lines upon CCL2 stimulation. Upon CCL2 treatment survivin expression was restored in shS-1 to similar levels as observed for parental cells (Fig. 9D) and consequently, cell survival was recovered, even though untreated shS-1 exhibited reduced viability when compared to control (parental) cells (Fig. 9, A-C). In contrast, CCL2 treatment only partially rescued the survivin levels in shS-2 cells (Fig. 9H), as a result, the cell viability was not restored upon CCL2-stimulation (Fig. 9F and 9G). Furthermore, the observed differences in viability between shS-2 and control cells increased with time (from 48 to 96 hours) in response to CCL2 stimulation. However, at 24 hours, when survivin expression is not yet highly affected by serum starvation (see Fig. 3A), survival was also unaffected by addition of CCL2 (Fig. 9E). Additionally, shS-1 and shS-2 (+/- CCL2) were analyzed by immunofluorescence microscopy 72 hours post-treatment using the LC3B antibody. As shown in Fig. 9I, the control shS-1 cells evidenced a remarkably higher punctate pattern as compared to CCL2-treated shS-1 cells. The observed difference is consistent with the rescue in survivin expression that occurred in shS-1 after CCL2 stimulation (Fig. 9D, lanes 1 & 2) and correlates with the survival differences between treated and untreated cells (Fig. 9B & 9C). In contrast, no major differences were noticed in the immunofluorescence pattern between control and CCL2-treated shS-2 cells (Fig. 9J). In this case both cell populations showed a similar punctate pattern, consistent with the inability of CCL2 to rescue both cell survival and survivin expression (Fig. 9H).

Therefore, survivin protein controls the cell viability and localization of LC3 as a result of its recruitment to the autophagic membranes,

suggesting that survivin is a critical factor that mediates the CCL2 protection from autophagic death upon serum starvation.

On the basis of these findings, it was hypothesized that survivin might control the extent of autophagy by interacting with LC3. Figs. 10a,b show the results of two independent immunoprecipitation experiments performed with control and CCL2-treated PC3 cell-extracts collected 48h post-treatment. The extracts were immunoprecipitated with survivin or non-specific rabbit IgG antibodies (at two different dilutions) and the immunoprecipitates were probed on Western blot for the presence of LC3B and survivin (positive control). Strong evidence for the association of survivin and LC3 was obtained. Both in control and CCL2 samples LC3 co-precipitated with survivin antibody. This interaction could be of vital importance in the survivin role of controlling autophagy and protecting the cells upon serum deprivation.

#### Discussion

Research in cancer biology has demonstrated that various host factors can have a profound effect on cancer behavior. These include inflammatory host responses and immune deficiencies that could reduce the inherent resistance to some cancers. CCL2 plays an important role in the inflammatory response by regulating the recruitment of monocytes/macrophages and other inflammatory cells to sites of inflammation. Several reports have implicated this chemokine in the host activities that impact cancer and have important effects on cancer progression and metastasis (38-42). Indeed, recent data have demonstrated that targeting the CCL2 chemokine with neutralizing antibodies induces prostate cancer tumor regression *in vivo* (9) and may represent a novel approach to cancer treatment. However, it has not been previously reported that this chemokine may function to directly promote cancer cell survival by protecting them from cell death. The data presented here, reveal that CCL2 provides a survival advantage to prostate cancer PC3 cells by inhibiting autophagic death, both through the activation of PI3K-Akt signaling pathway and up-regulation of survivin.

Upon serum deprivation, cell survival increased in response to CCL2 treatment reaching

a maximum response at 50-100ng/ml. By measuring cell viability at different times and cell cycle analysis of control and CCL2-treated cells (Fig. 5), it was concluded that PC3 cells are unable to proliferate under serum-free conditions; rather, they exhibit a prolonged resistance to death (> two weeks) when treated with CCL2. Moreover, upon re-addition of serum, CCL2-treated cells restored the ability to proliferate, even after four weeks in serum-free conditions (see Fig 1E).

A major signaling pathway induced by CCL2 in these cells is the PI3K-Akt pathway. The Akt serine/threonine kinase is a central node in the signaling downstream of growth factors, cytokines and other cellular stimuli. Several reports have linked Akt to cell survival and inhibition of both apoptotic and non-apoptotic cell death (23,43). It is also possible that Akt exerts some of its survival effects through crosstalk with other pathways and it is well known that Akt activates mTOR and vice versa (44,45). CCL2 stimulation of PC3 cells, upon serum deprivation, results in Akt hyperphosphorylation of the two key regulatory sites (Ser<sup>473</sup> and Thr<sup>308</sup>) required for its full activation. Furthermore, blocking this phosphorylation with the PI3K inhibitor LY294002, or the Akt-specific inhibitor Akti-X, abrogated the CCL2-mediated cell survival.

An important result from the current study is the finding that CCL2 maintains survivin expression by up-regulating both mRNA and protein upon serum deprivation. CCL2-induced survivin up-regulation could be explained, at least in part, by CCL2-dependent transcriptional regulation of this gene, as demonstrated by Real-Time PCR (see Fig. 3B). However, the possibility that CCL2 signaling could also regulate survivin post-transcriptionally can not be completely ruled out, and future investigations will be needed to address this mechanism.

Numerous reports indicate that survivin expression inhibits cell death induced by various apoptotic stimuli *in vitro* (25,46,47) and *in vivo* (16,27,28). In addition, the Akt pathway has been previously reported to be linked with survivin up-regulation as well as protection of prostate cancer cells from apoptosis (15). Here, CCL2-mediated survivin up-regulation was shown to be PI3K-Akt dependent; inhibition of this pathway not only abrogated survivin expression, but also dramatically decreased cell survival. On the other

hand, no significant changes in expression were observed for other members of the IAP-family proteins. These results indicate a strong link between PC3 cell survival and survivin regulation. This study also shows that upon serum deprivation, survivin is up-regulated by CCL2 in other prostate cancer cells: C4-2B and DU145, suggesting an analogous survival mechanism in these cells.

Further significance of survivin overexpression in cancer cells comes from the evidence that it is normally expressed during embryonic and fetal development but undetectable in most normal adult tissues (48). The promoter of the survivin gene is specifically induced during the G2/M phase of the cell cycle (49). However, immunohistochemical analysis of tumor samples indicates that survivin may actually be expressed continuously in a cell-cycle independent manner, consistent with the evidence that it is deregulated in cancers. In PC3 cells, CCL2-mediated survivin expression was shown to be cell-cycle independent; after serum-starvation, cells are arrested mostly in G1 and no cell cycle progression is apparent (see Fig. 5). Moreover, survivin becomes the fourth most expressed transcript in human cancer (50) where it correlates with a more aggressive disease and reduced overall survival (27).

The data presented here indicate that apoptosis is not the major cell death mechanism induced in serum-deprived PC3 cells. In fact, FACS analysis of control and CCL2-treated cells evidenced very low apoptosis (see Fig. 5) and no substantial differences were noticed between the two cell populations (+/- CCL2). These results are consistent with previous reports, showing that in PC3, endogenous Bcl-XL overexpression provides a survival mechanism that protects these cells from apoptotic signals emanating from PI3K inhibition (29).

On the basis of these findings, it was hypothesized that serum-deprived PC3 cells were dying by autophagy. During periods of nutrient shortage, autophagy plays a critical protective role, providing the constituents required to maintain cellular metabolism and could promote cancer cell survival under metabolic stress (51-53). In most situations, autophagy likely functions initially as a cytoprotective mechanism, but if cellular damage is too extensive, or if apoptosis is compromised,

excessive autophagy may actually kill the cell (54-56). It was shown here that autophagy is hyper-induced in these cells in response to serum-deprivation. In fact, the amount of LC3-II (an indicator of autophagosome formation) increased dramatically within the first 24 hours of serum starvation. However, because an increase in autophagy also results in rapid LC3-II degradation, it could lead to misinterpretation of the immunoblot results; therefore, autophagic degradation was evaluated in the presence of protease inhibitors. Here, it was demonstrated that by adding E64D and Leupeptin, LC3-II further increased, indicating that autophagic degradation occurs in both control and CCL2-treated cells (Fig. 6A); consequently, the higher LC3-II detected in control cells (Figs. 6Ba,b) can not be attributed to an inhibition of autophagic degradation, but rather an increase in the autophagosome formation. On the other hand, in CCL2-stimulated cells, the relative number of autophagosomes (LC3-II) increased when treated with the PI3K or Akt specific inhibitors: LY294002 and Akti-X (Figs. 6Ca,b and 6D), correlating with a decrease in cell viability (see Figs. 2B & 2C). Additionally, the immunofluorescence analysis of LC3 localization evidenced a remarkably higher LC3 punctae in control as compared with CCL2-treated cells (Fig. 6E), consistent with LC3-II protein levels detected by Western blot. Altogether, these results suggest that CCL2 signaling limits overall autophagy and, at least partially, protects PC3 cells from death by inhibiting the autophagosome formation.

CCL2 protection from autophagic death was further elucidated by treatment of PC3 cells with rapamycin. Autophagy is controlled by pathways that impinge on the mammalian target of rapamycin (mTOR). Activation of mTOR inhibits autophagy and therefore, treatment with rapamycin induces autophagy (17,33-35,57). The data presented here demonstrated that rapamycin further induced cell death 24 hours post-treatment and CCL2 significantly protected cells from rapamycin-induced death (Fig. 7Aa,b). Furthermore, treatment with rapamycin significantly down-regulated survivin expression (see Fig. 7B), thus further suggesting a link between PC3 cell survival and CCL2 protection from autophagic death, via survivin up-regulation. Moreover, several reports indicate that PI3K-Akt signaling inhibits autophagy (58,59) and mTOR is

regulated through the PI3K-Akt pathway (60). CCL2, but not IGF-1, induced significantly higher phosphorylation of p70S6K at Thr<sup>389</sup>, detected even at 48 and 72 hours post-treatment (Fig. 7D). This phosphorylation was blocked by Akti-X (Fig. 7C), correlating with lower survivin expression and higher LC3-II levels, thus pointing out the crucial role of Akt-mTOR activation in this survival mechanism.

CCL2-mediated survival was substantially reduced by *Survivin*-specific siRNA, compared to control siRNA. Similarly, shS-1, a stable cell line depleted of survivin, exhibited approximately 50 % reduction in cell viability (at 96 hours) as compared to parental PC3 cell line. Stimulation by CCL2, however, rescued the survivin expression levels and, as a result, viability was also restored (Figs. 9A-9D). A different result was observed for the survivin-depleted shS-2 stable cell line. Here, the viability of shS-2, as compared to control cells, was slightly lower only at early times (24 and 48 hours; see Figs. 9E and 9F), however, by 96 hours (Fig. 9G), no substantial differences were observed between shS-2 and control cells. This result could be explained by the pattern of survivin expression – indeed, survivin expression dropped dramatically after 48 hours (see Fig. 3A), consequently, by 96 hours, the survivin depletion by shRNA has insignificant effect. On the other hand, when these cells (shS-2 and control) were stimulated with CCL2, the difference in cell viability became evident at 48 hours and further increased by 96 hours (Figs. 9F and 9G). These findings are consistent with the depletion of survivin observed at 48 hours in CCL2-treated shS-2 cells, with respect to CCL2-treated control cells (Fig. 9H, lanes 3 & 4). Furthermore, the findings for LC3 localization by immunofluorescence microscopy correlated with the cell viability and survivin protein levels of shS-1 and shS-2. In fact, LC3 localization (punctate pattern) in shS-1 revealed striking

differences when the cells were treated or not with CCL2 (Fig. 9I); a response similar to that observed in parental PC3 cells (Fig. 6E). In contrast, shS-2 cells did not exhibit a different pattern of LC3 localization in response to CCL2 stimulation (Fig. 9J). Altogether, these experiments reveal a strong correlation between cell viability, LC3 localization and survivin expression in PC3 cells upon serum deprivation. Furthermore, recently LC3 was shown as a mediator of tethering and hemifusion of autophagosome membranes (61)), and here, strong evidence for interaction between survivin and LC3 was detected by co-immunoprecipitation experiments (Fig 10), suggesting an important link that could be essential in the protection mechanism exerted by CCL2. However, this interaction deserves further investigation to define the exact role of survivin in the control of autophagy.

The proposed mechanism for CCL2-mediated inhibition of autophagic death is described in Fig. 11: CCL2 activates PI3K-Akt pathway which in turn mediates the activation of mTORC1, up-regulation of survivin and down-regulation of autophagosome formation, thus protecting PC3 cells from autophagic death. Conversely, rapamycin down-regulates survivin and hyper-activates autophagy resulting in increased cell death. This paper, for the first time, reveals survivin as a critical molecule that protects cells from autophagic death. By inhibiting the two major programmed cell-death mechanisms, apoptosis and autophagy, survivin becomes a crucial protein that allows cancer cells to survive many cell-death stimuli including nutrient starvation. Accordingly, by up-regulating survivin, CCL2 could suppress both autophagy and apoptosis in cancer cells. In view of our results, we propose combined therapies targeting CCL2 and PI3K/Akt/mTOR/survivin pathway as a valid strategy to overcome prostate cancer cells resistance to death.

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## FOOTNOTES

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## FIGURE LEGENDS

**Fig. 1.** CCL2 signaling prolongs survival in serum-starved PC3 cells, *in vitro*. (A) PC3 cells were synchronized by serum-starvation for approximately 24 hours then treated with increasing concentrations

of CCL2 to assess the dose-dependent effects from 0 to 100 ng/ml. Cell Viability was evaluated by WST-1 dye conversion at 24 hour increments for 96 hours and then at 1 week. All absorbencies were normalized to untreated (control) samples at the 24 hour time point. (B) LIVE/DEAD<sup>®</sup> cell viability assay of both CCL2 (100 ng/ml) treated and untreated PC3 cells at 72, 96, and 168 hours. Viable cells fluoresce at 515nm (green emission wavelength) and dead cells fluoresce at 635nm (red emission wavelength). (C) Quantification of live and dead PC3 cells over time, in response to CCL2 (100ng/ml) treatment, data shown represents average quantities from six fields at each time point (Based on LIVE/DEAD assay, Fig. 1B). (D) PC3 cells were plated into dual-well chamber slides (Lab-tek II, Nunc) in serum-free RPMI 1640 media. One chamber of each slide was treated with CCL2 (100 ng/ml) and cells were incubated without changing media or further CCL2 supplementation. Following incubation for 14 days, cells were observed using an Olympus FV-500 Confocal microscope. Two representative DIC images for both control (a,b) and CCL2-treated (c,d) cells are shown here. (E) Cells were treated as described in Fig. 1D, however, cells shown in the DIC image above were incubated for 4 weeks in serum-free media. Following incubation, cells were fed by replacing media with RPMI 1640 + 10% FBS. 72 hours after addition of serum, recovery was observed in CCL2-treated cells; selections from this field have been magnified (c,d) to display cells showing normal PC3 morphology. No viable cells were observed in untreated PC3, following addition of FBS (left image). Cell viability was demonstrated also by WST-1 analysis; corresponding WST-1 absorbance values are shown below each image. (F) Cell survival curve representing PC3 response to treatment with increasing concentrations of recombinant IGF-1, based on WST-1 dye conversion. Values represent average absorbencies of n=8 samples, normalized to untreated cells at t=24h. IGF-1 survival was also compared to PC3 cells treated with two concentrations of CCL2 (25 and 100 ng/ml).

**Fig. 2.** CCL2-mediated cell survival is PI3K-Akt pathway-dependent. (A) Immunoblot analysis of the time-dependent effect of CCL2 (100 ng/ml) on Akt phosphorylation in serum-starved PC3 cells, using: (a) anti-P-Ser<sup>473</sup>Akt and (b) anti-P-Thr<sup>308</sup>Akt rabbit antibodies (Cell Signaling; 9271 and 9275, respectively). Phosphorylation specificity was evaluated by comparison to total Akt expression for each blot.  $\beta$ -actin was used as a protein loading control for all blots. (B) CCL2 elicits cell survival through a PI3K-Akt dependent mechanism. Serum-starved PC3 cells were treated with increasing concentrations of PI3K-Akt inhibitor (LY294002), in the presence or absence of CCL2 (100 ng/ml). Cell survival analysis, based on WST-1 dye conversion was performed at 24 hour increments over a 96 hour time-course experiment. (a) Values depicted in the survival curve above represent average absorbencies of n=5 samples, normalized to untreated cells at t=24h. (b) Akt phosphorylation at both the Thr<sup>308</sup> and Ser<sup>473</sup> sites in PC3 cells treated with CCL2 (+/- LY294002 (2 $\mu$ M)), was evaluated by immunoblot analysis of cell lysates isolated at increasing post-treatment times. Phosphorylation was validated by comparison to total Akt expression. (C) Akt inhibitor-X (Akti-X; Calbiochem, 124020) reduces cell survival over time, in response to serum-starvation, and prevents Akt phosphorylation at both the Thr<sup>308</sup> and Ser<sup>473</sup> sites. Serum-starved PC3 cells were treated with the indicated concentrations of Akti-X  $\pm$  CCL2. (a) Cell survival was evaluated at 24-hour increments for 96 hours by WST-1 dye conversion. The survival curves shown above represents average absorbencies (440nm) of n=5 samples, normalized to untreated cells at t=24h. (b) Akt phosphorylation for treated and untreated PC3 cells  $\pm$  CCL2 (100 ng/mL), over time, was evaluated by immunoblot analysis and verified by comparison to total Akt expression.

**Fig. 3.** CCL2 up-regulates survivin mRNA and protein expression via a PI3K-Akt dependent mechanism. (A) Immunodetection showing the effect of CCL2 (100 ng/ml) on IAP family proteins in treated and untreated PC3 cells, over time. (B) Real-time PCR analysis of RNA samples, isolated from serum-starved PC3 cells, 72 hours following CCL2-treatment. Relative *Survivin* mRNA expression levels represent n=3 samples. *Survivin* up-regulation, in response to CCL2, was also confirmed by immunoblot analysis of protein lysates collected 72 hours post-treatment. (C) LY294002 specifically abrogates endogenous survivin expression (with respect to other IAP proteins) in serum-starved PC3 cells, over time, and prevents rescue by treatment with CCL2, as shown by immunoblot analysis. (D)



Immunodetection of protein lysates from serum-starved PC3 cells, treated with increasing concentrations of Akti-X, in the presence of CCL2 (100 ng/ml) at (a) early and (b) late time points. (E) The effect of IGF-1 (100 ng/ml) on Akt phosphorylation and survivin expression in serum-starved PC3 cells over time, as determined by immunodetection.

**Fig. 4.** CCL2 signaling induces survivin up-regulation in other prostate cancer cells in response to serum-starvation. Immunoblot analysis of survivin expression (+/- CCL2 (100 ng/ml)) at increasing times post-treatment, in (a) C4-2B and (b) DU 145 serum-starved cells.

**Fig. 5.** Cell cycle analysis of serum-starved control and CCL2-treated cells. FACS analysis of serum-starved PC3 cells  $\pm$  CCL2 (100 ng/ml) based on PI staining at increasing collection times (48-120 hours). DNA histograms for each specified time point are representative of three independent cell cycle analyses. Corresponding values for each cell population ( $G_0/G_1$ , S, and  $G_2/M$ ) are expressed as percentages of cells found within each phase of the cell cycle. Sub  $G_0/G_1$  populations (apoptotic cells) were also determined and displayed as relative percentages.

**Fig. 6.** Autophagy is activated in PC3 cells upon serum deprivation and CCL2 regulates the autophagosome formation. (A) Immunoblot analysis examining starvation-induced autophagosome formation in time, via LC3-II expression, using an anti-LC3B antibody (Cell Signaling; 2775). Six hours following attachment in serum-free media, synchronized PC3 cells were treated with or without CCL2 (100 ng/ml). Protease inhibitors Leupeptin (Calbiochem; 108975) and E64D (Calbiochem; 330005) were also added to the medium where indicated (10  $\mu$ g/ml, each). As a control for non-starved cells, protein lysate was isolated from cells grown in serum-containing (10% FBS) medium. Both LC3-I (upper band) and its phosphatidylethanolamine-conjugated form, LC3-II (lower band) are detected by this LC3B antibody. (B) (a) Protein lysates from control and CCL2-treated cells  $\pm$  protease inhibitors, described in A, were run in adjacent wells, prior to immunodetection to accurately compare the LC3-II bands at 24 and 72 hours. FBS samples were used as a control for non-serum-starved (low autophagy) cells. (b) Fold increase in LC3-II expression in untreated versus CCL2-treated cells at 24 and 72 hours, post-treatment was quantified and evaluated by densitometric analysis with  $n=3$  samples, using FluorChem HD2 software (Alpha Innotech). (C) (a) The effect of the PI3K inhibitor, LY294002 (LY), on LC3-II expression over time, in CCL2-treated cells was determined by LC3B immunoblotting. (b) LC3-II Fold increase in CCL2+LY versus CCL2-treated cells at 24, 48, and 96 hours post-treatment was determined by densitometric analysis with  $n=5$  samples. For all densitometric quantifications, expression values were normalized to  $\beta$ -actin. (D) The effect of Akt inhibitor-X (Akti-X) on endogenous LC3-II, in CCL2-treated cells at 48 and 72 hours post-treatment. (E) Localization of endogenous LC3 upon serum starvation in control and CCL2-treated PC3 cells. The indicated cells were treated or not with CCL2 (100 ng/ml) for 72 hours and subjected to immunofluorescence microscopy using a selective antibody against LC3B (Cell Signaling), in combination with goat-anti-rabbit AlexaFluor 488-conjugated IgG, (Invitrogen, #A11034). Maximum intensity projections from Z-stacks obtained by confocal microscopy are shown for (a) control, and (c) CCL2-treated PC3 cells. DIC images of corresponding fields are shown to the right of respective fluorescent images (b,d).

**Fig. 7.** CCL2 protects PC3 cells from autophagic death. (A) Synchronized PC3 cells were plated in serum-free media for 24 hours prior to treatment with increasing concentrations of Rapamycin (0-40 nM)  $\pm$  CCL2 (100 ng/ml). Cell viability was assessed by WST-1 dye conversion, 24 hours post-treatment (a) The survival curves represents the average absorbencies (440 nm) of  $n=5$  samples, for each condition  $\pm$  CCL2 (100 ng/ml), in response to increased rapamycin concentration. (b) The data represented in "a" were normalized to 0 nM rapamycin for each condition (+/- CCL2), and are expressed as relative percent survival. (B) A subset of cells treated as described in "a"  $\pm$  20 nM rapamycin were analyzed by immunoblotting using survivin antibody. (C) The effect of Akt inhibitor-X (Akti-X) on phospho-p70S6 kinase (Thr<sup>389</sup>) in CCL2-treated cells at 48 and 72 hours post-treatment by Western blot analysis as

compare with LC3B and survivin protein levels. (D) IGF-1 (100 ng/mL) induces p70S6K phosphorylation (Thr<sup>389</sup>) only at early time points in PC3 cells as determined by Western blot analysis of cell lysates as indicated.  $\beta$ -actin was used as a loading control.

**Fig. 8.** Analysis of PC3 cell survival in CCL2-treated cells transfected with survivin siRNA. Serum-starved PC3 cells were transfected as described in materials and methods with either control (non-silencing) or *Survivin*-specific siRNA at concentrations of 50nM and 100nM, as indicated. 24 hours following transfection, cells were either treated or not with CCL2 (100 ng/mL). (A) Data represents average WST-1 absorbencies (440 nm) obtained from n=3 samples  $\pm$  CCL2, for each transfection condition, 72 hours post-treatment. Data shown has been normalized to control (non-silencing) siRNA-transfected cells; 72 hours post CCL2 treatment. P-values for survivin versus control are shown above respective CCL2 samples. (B) Immunoblot analysis of control (non-silencing) and survivin siRNA-transfected PC3 cells at 24, 48, and 72 hours, post-transfection. Protein lysates were probed with survivin antibody.  $\beta$ -actin antibody was used as a loading control.

**Fig. 9.** Analysis of CCL2-mediated survival and LC3 localization in shS-1 and shS-2 stable cell lines. (A-C) Cell viability of serum-starved parental PC3 (control) and stable-transfected PC3 cells expressing survivin shRNA, “shS-1” (Open Biosystems, V2LHS\_94585), in response to treatment with CCL2 (100 ng/ml) was assessed by WST-1 dye conversion at: (A) 24 hours, (B) 48 hours, and (C) 96 hours. Data shown represents the average absorbencies (440 nm) + SE from n=5 samples for each group  $\pm$  CCL2. (D) Survivin expression analysis by Western blot of control (parental) and shS-1 stable-transfected cells  $\pm$  CCL2, 48 hours post-treatment.  $\beta$ -Actin was used as a loading control as indicated. (E-H) The experiments described in “A-D” were repeated using a second stable-transfected PC3 cell line expressing shS-2 (Open Biosystems, V2LHS\_262484). Figures “E-H” correspond to images “A-D”, respectively. (I,J) Localization of endogenous LC3 upon serum starvation in control (panel a) and CCL2-treated (panel b) cells: (I) shS-1 and (J) shS-2. The indicated cells were treated or not with CCL2 (100 ng/ml) for 72 hours and subjected to immunofluorescence microscopy using a selective antibody against LC3B. AlexaFluor 647-conjugated goat-anti-rabbit IgG (Invitrogen, #A21245) was used as the secondary antibody for the immunofluorescence detection of LC3B. Maximum intensity projections from Z-stacks obtained by confocal microscopy are shown for (a) control, and (b) CCL2-treated shS-1 or shS-2 cells. Selections from these fields have been magnified to display the LC3 punctate patterns.

**Fig. 10.** Protein-protein interactions between survivin and LC3B. Two independent immunoprecipitation experiments performed with control and CCL2-treated PC3 cell-extracts collected 48h post-treatment. The extracts were immunoprecipitated with survivin or non-specific rabbit IgG antibodies (Cell Signaling) at two different dilutions: (a) 1:100 and (b) 1:200. The immunocomplexes were probed on Western blot for the presence of LC3B and survivin. Immunodetection for survivin was performed to verify immunoprecipitation efficiency and specificity; corresponding blots are shown directly below respective LC3B blots. Input samples for both control and CCL2 lysates are also shown for each immunoblot.

**Fig. 11.** Proposed mechanism by which CCL2 induces PC3 cell survival upon serum-starvation. CCL2 activates PI3K-Akt pathway which in turn mediates the activation of mTORC1, up-regulation of survivin and down-regulation of autophagosome formation, thus protecting PC3 cells from autophagic death. Conversely, rapamycin down-regulates survivin and hyper-activates autophagy resulting in increased cell death.

Figure 1

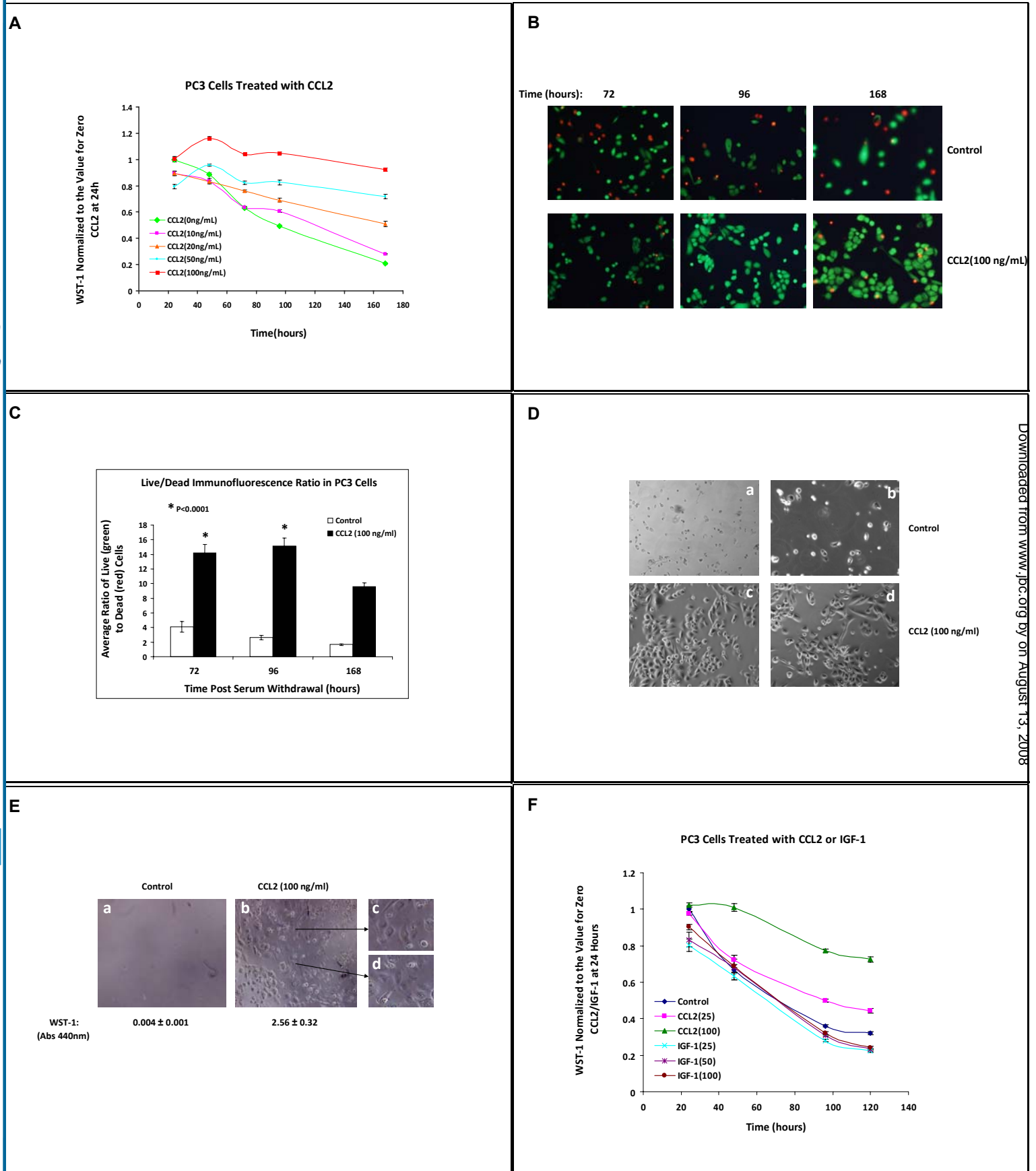


Figure 2

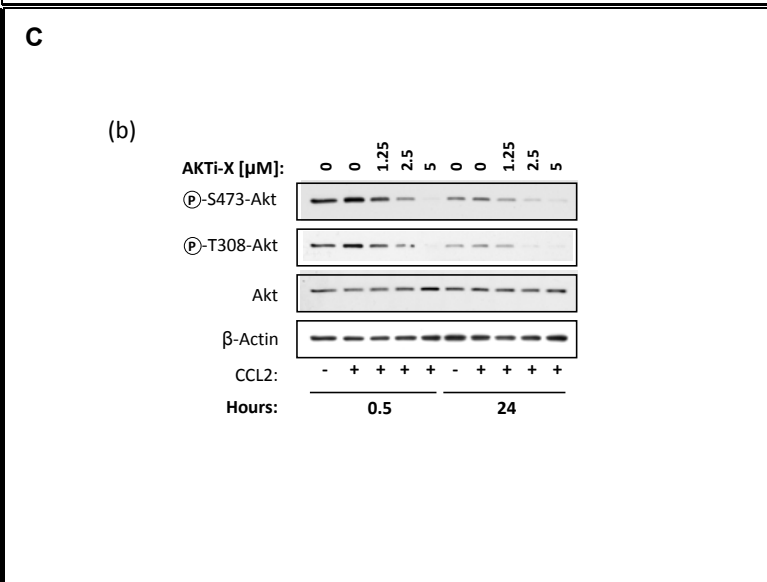
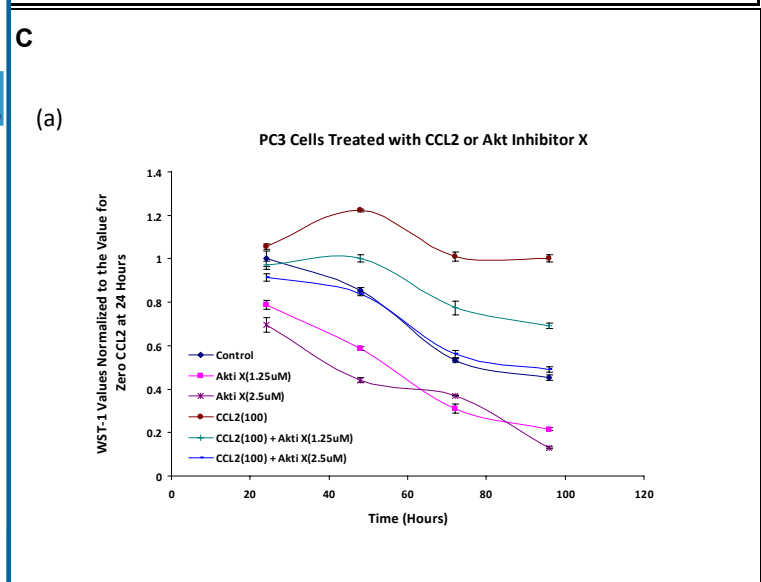
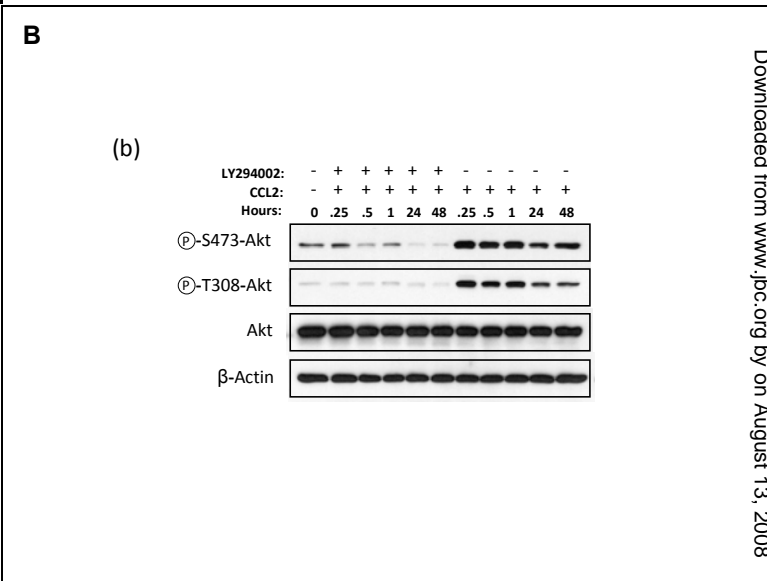
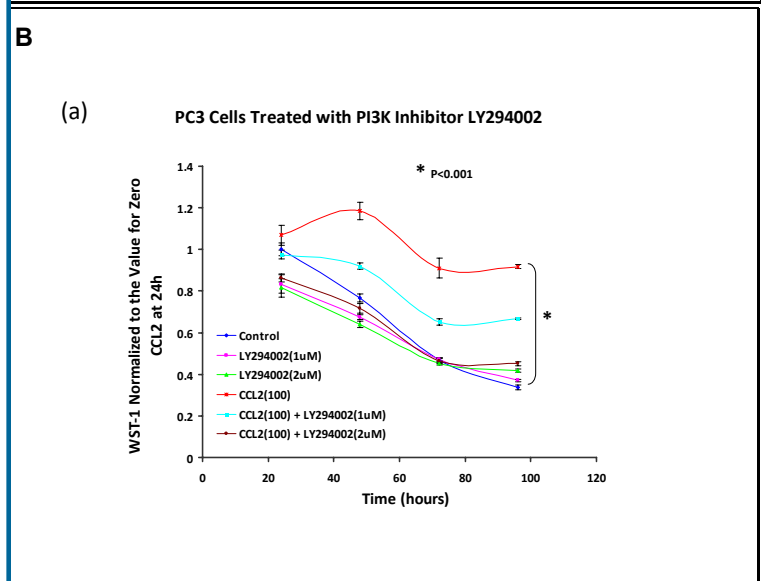
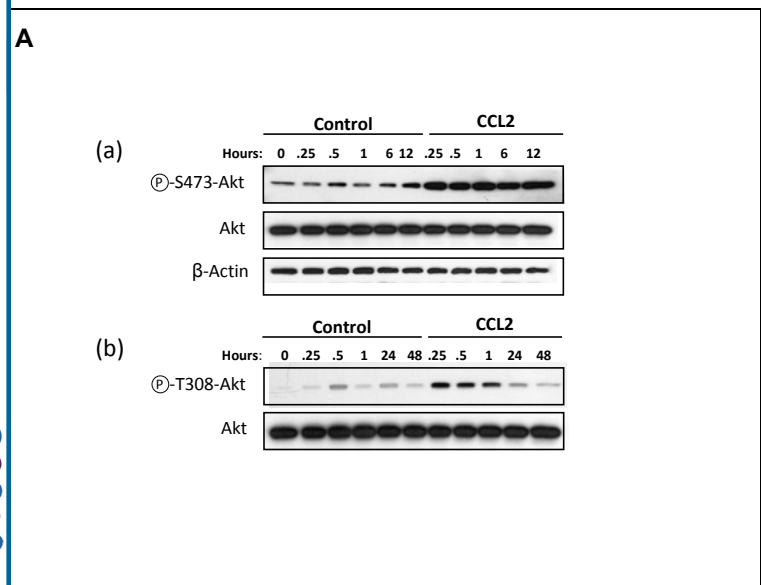




Figure 3

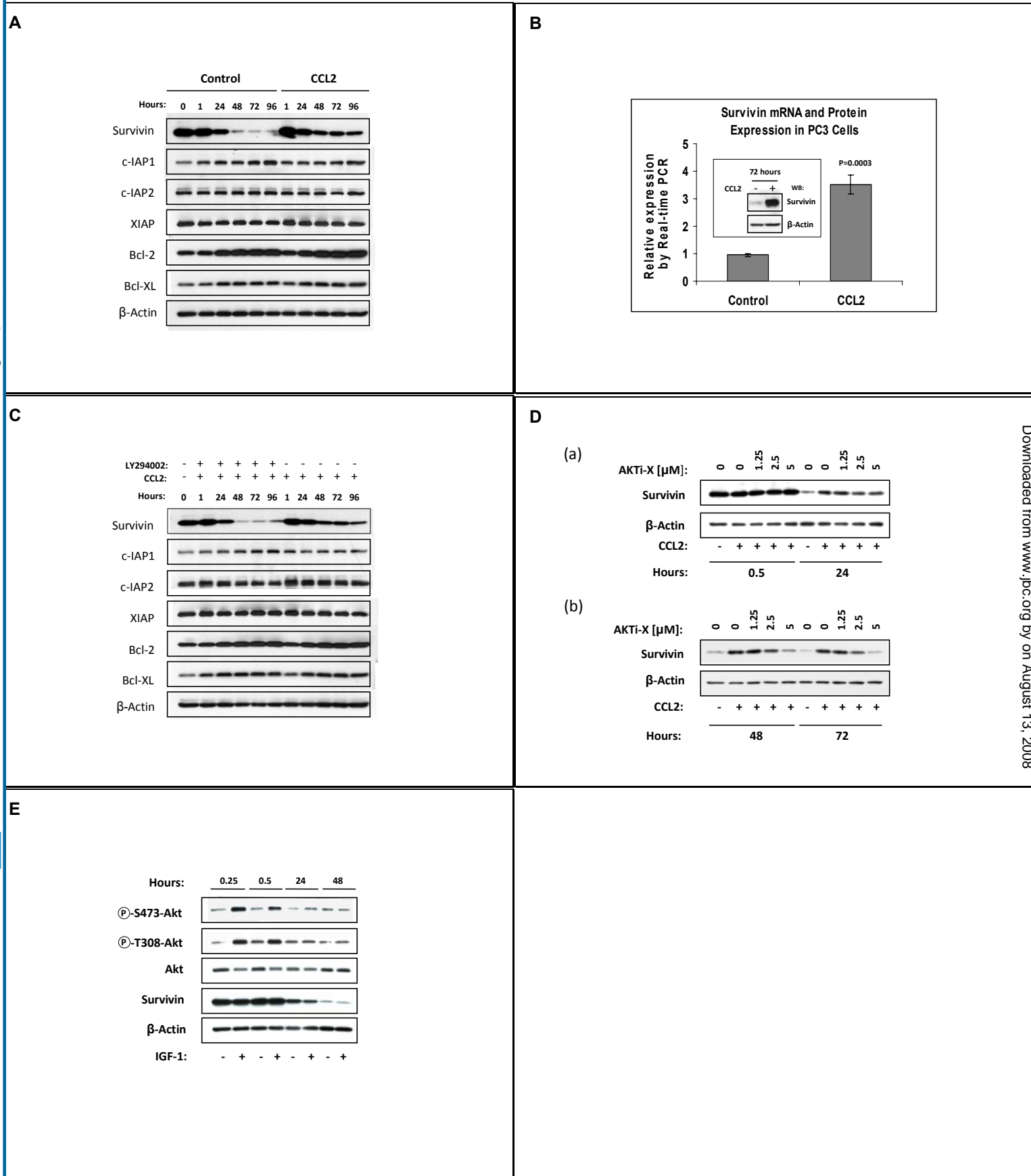


figure 4

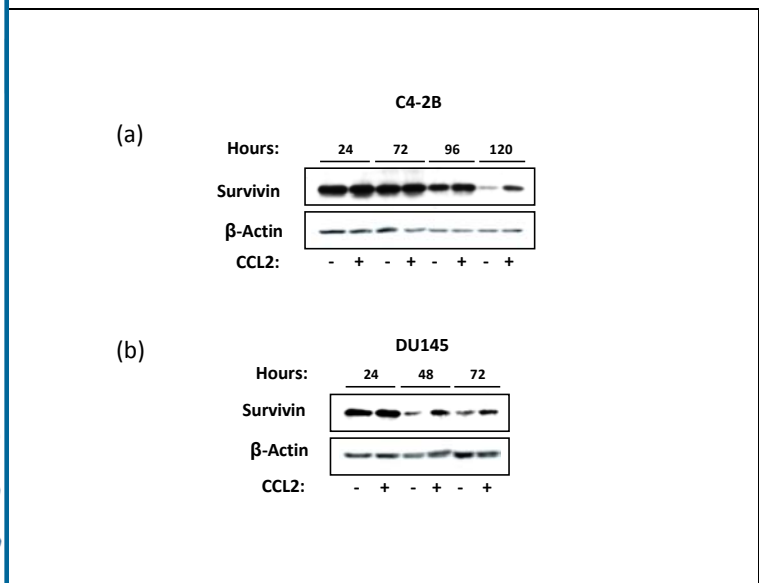


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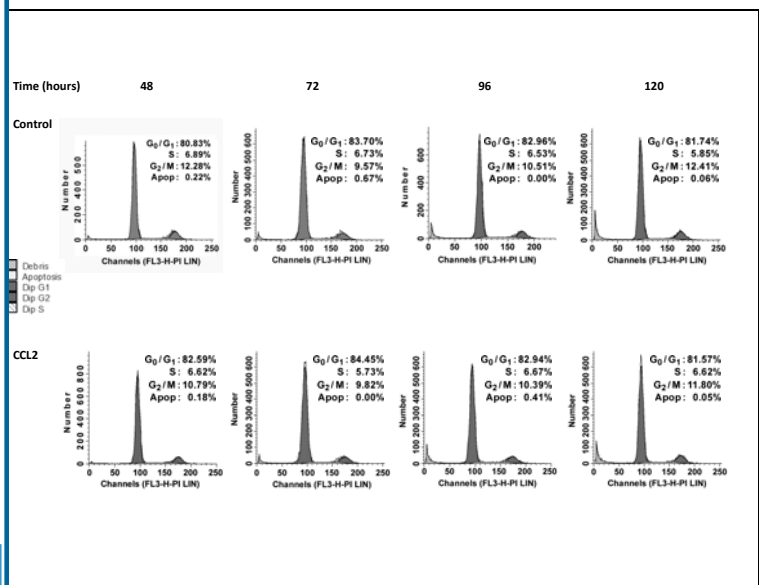


Figure 6

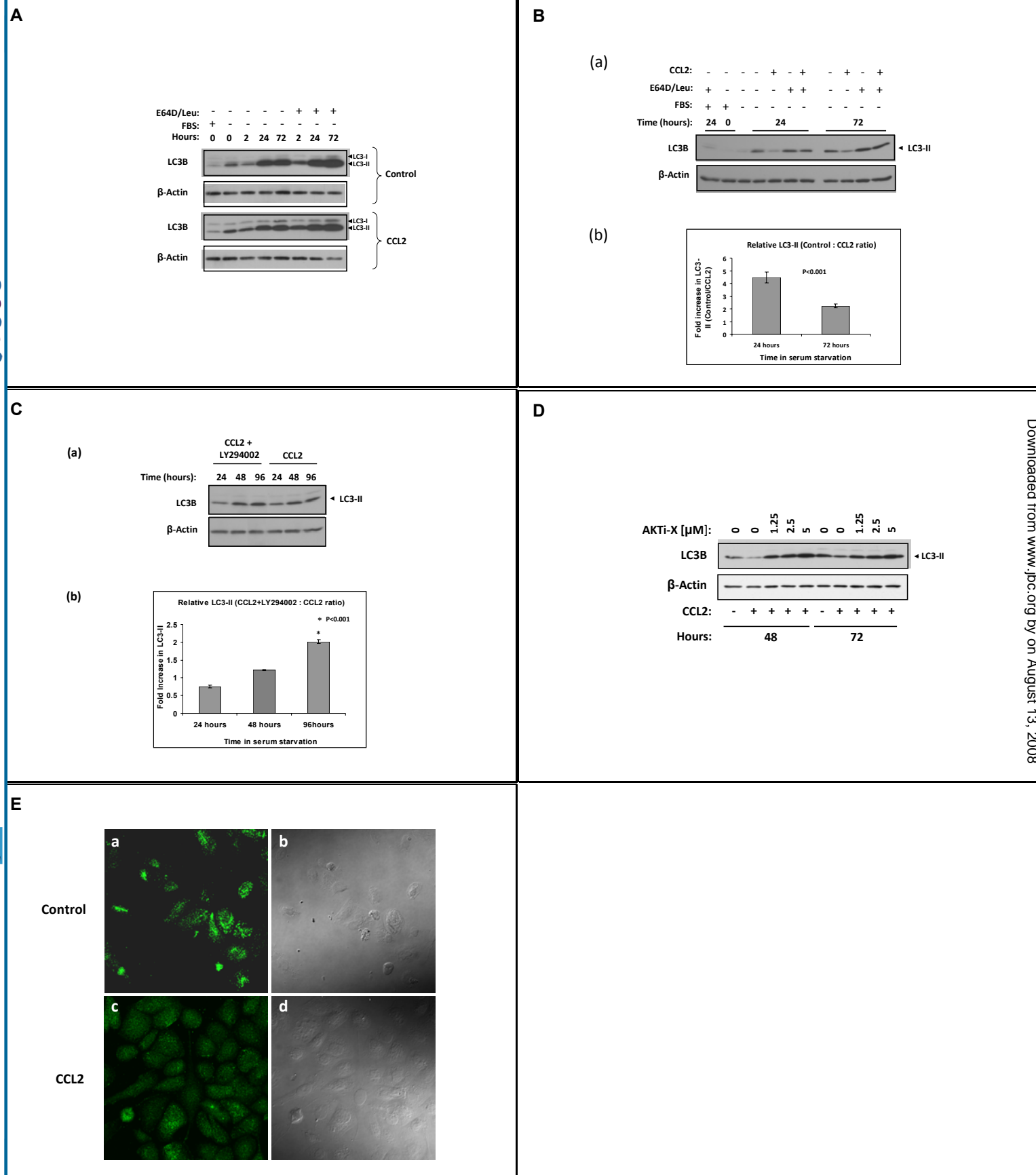


Figure 7

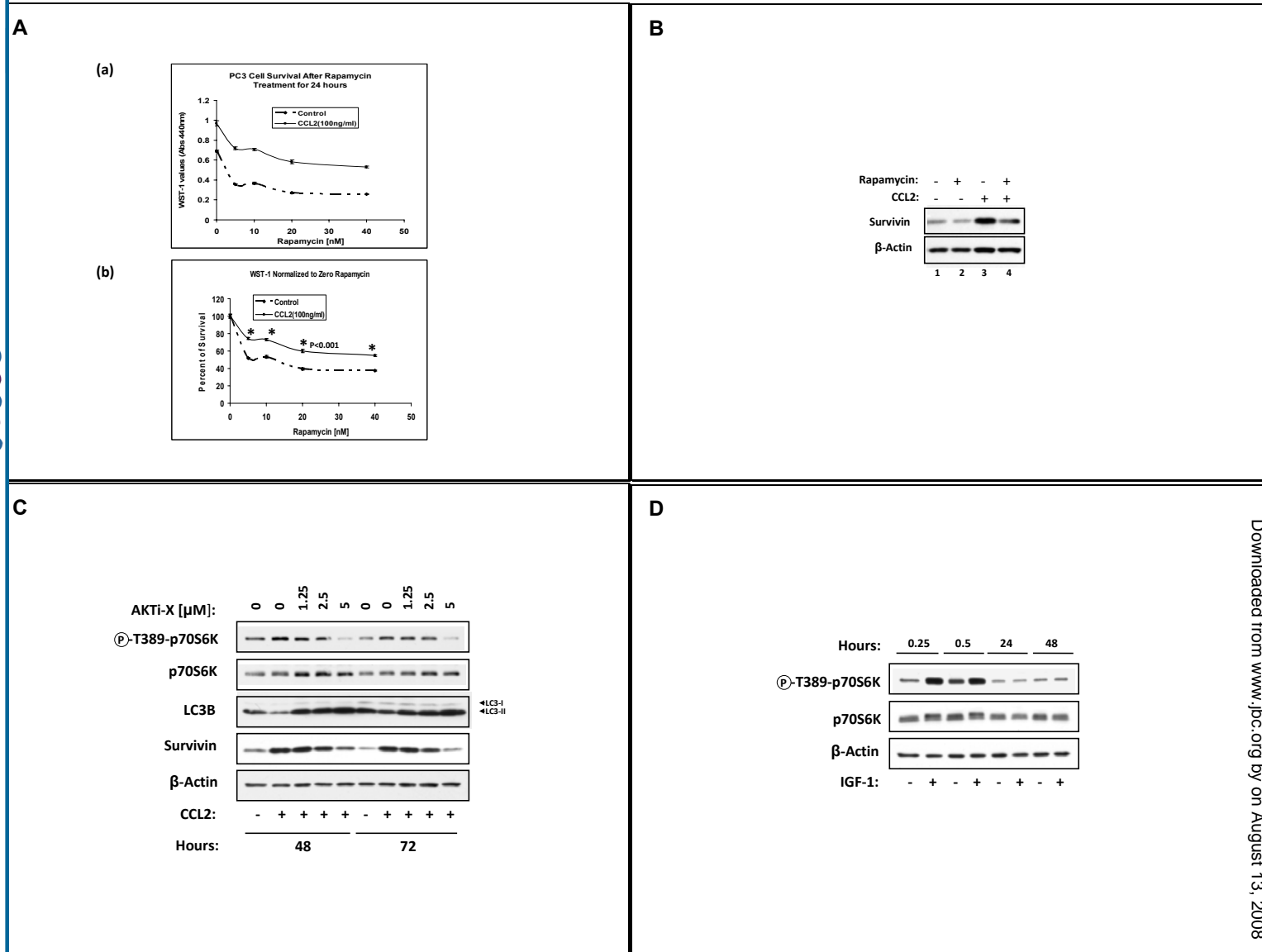
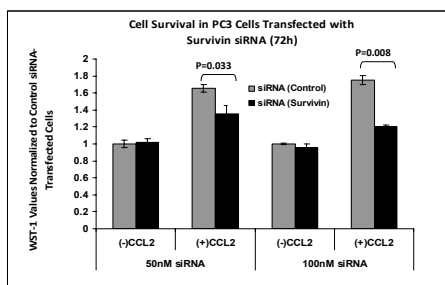




Figure 8

A



B

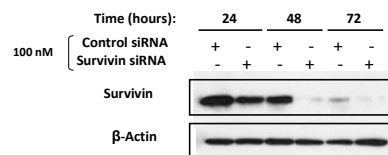
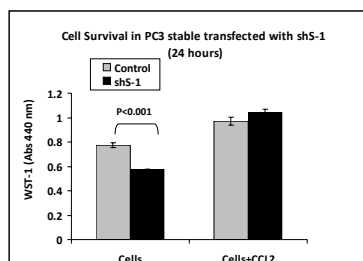
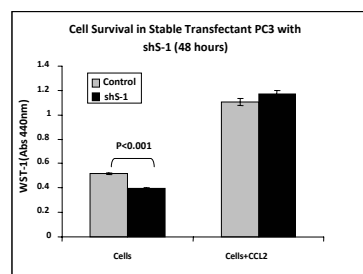


Figure 9

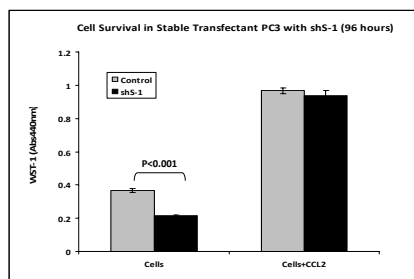
A



B

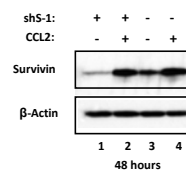


C

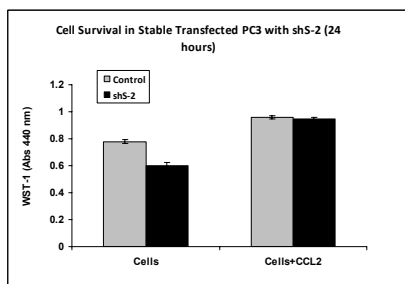


(Control +CCL2)/Control= 2.63  
(shS-1 +CCL2)/shS-1= 4.38

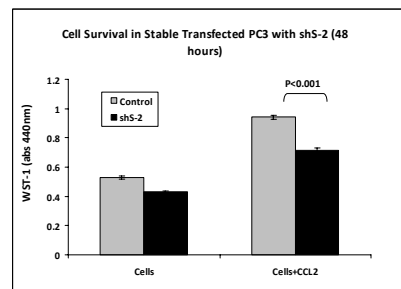
D



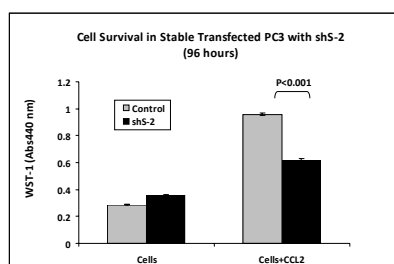
E



F

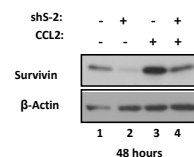


G

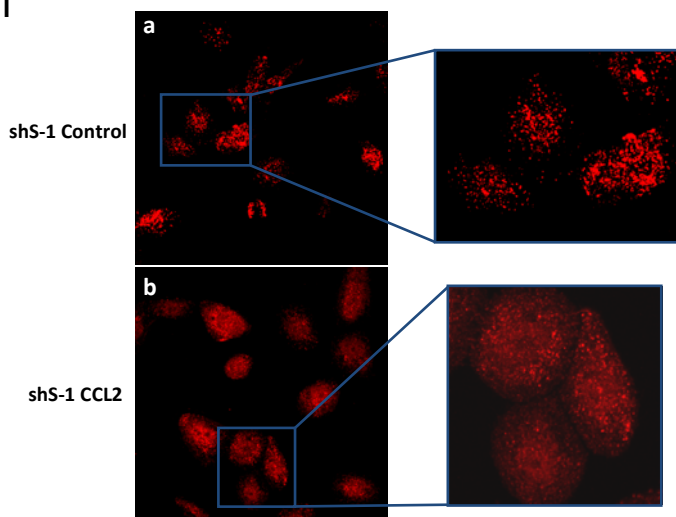


(Control+CCL2)/Control= 3.33  
(shS-2+CCL2)/shS-2= 1.75

H



I



J

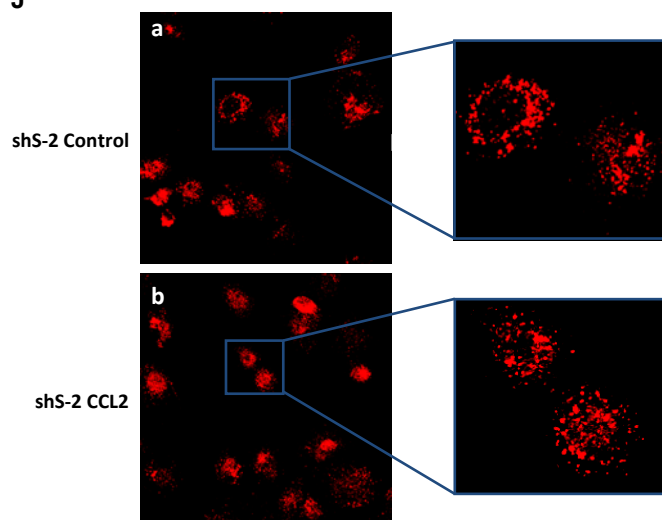


Figure 10

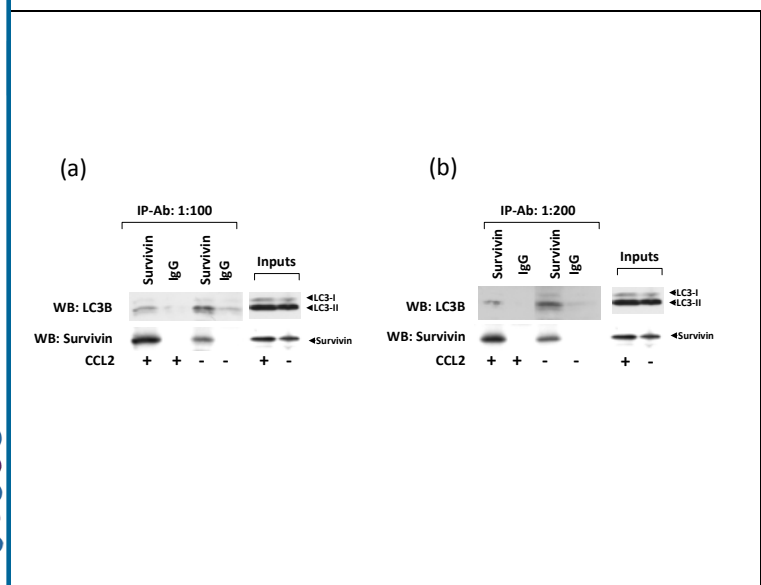


Figure 11

