Supplement Material and methods

Microarray analysis: RNA was isolated from PC3 cells following the transfection with siVEGF-C, siNRP-2 and scrambled siRNA. Duplicate sets for each condition were used for microarray analysis. The GCRMA method was used for background correction with quintile normalization for pre-processing of microarray data. The selection criteria for identification of differentially expressed genes were two fold expression change between groups of samples and FDR-corrected p-value of 0.05 or less.

Primer sequences for RQ-PCR:

VEGF-C forward: 5′- AGT GTC AGG CAG CGA ACA AGA -3′,
VEGF-C reverse: 5′- CCT CCT GAG CCA GGC ATC TG -3′;
NRP-2 forward: 5'-GTG AAG AGC GAA GAG ACA ACC A-3'.
NRP-2 -reverse: 5'-GCT GCA GTT CTC CCC ACA CT-3';
36B4 forward: 5'-ATG CAG CAG ATC CGC ATG T-3',
36B4 reverse: 5'-TCA TGG TGT TCT TGC CCA TCA-3'),

Methodology for RQ-PCR:

Quantiative real-time PCR (qRT-PCR) analyses were performed in an Applied Biosystems machine using Power SYBR®Green master mix with 75 ng cDNA and 50 nM forward and reverse primers in a final reaction volume of 25microliter. Specific primers were designed using Primer Express software (version 1.0; ABI). For relative quantification, gene profiles were normalized to the expression level of 36B4 for comparative Ct value analysis. Detail description of the microarray analysis has been provided in the supplement document.

Legends for Supplemental Figures

Supplemental Figure 1: Analysis of VEGF-C and NRP-2 expression following depletion by siRNA. PC3 cells were seeded into 6-cm dishes and treated with scrambled and either VEGF-C or NRP-2 siRNAs. 72 hours post-transfection, mRNA and protein were harvested from the cells for analysis. A, mRNA was reverse transcribed and the resulting cDNA was used in qPCR analysis with SybrGreen and VEGF-C-specific primers. The mRNA level was then calculated relative to 36B4 levels. B, Since VEGF-C is a secreted protein, the conditioned media was collected and analyzed via ELISA with a VEGF-C sandwich ELISA kit. TO determine the amount of VEGF-C present, the values obtained were compared to a standard curve. C, qPCR analysis of NRP-2. The level of NRP-2 was determined relative to the level of 36B4 mRNA. D, Immunoblot analysis of NRP-2 expression following treatment with either scrambled or NRP-2-specific siRNA.

Supplemental Figure 2: qPCR and ELISA Analysis of VEGF-C expression following treatment with specific siRNAs, siVEGF-C-1 and siVEGF-C-2. PC3 cells were treated with two individual siRNAs against VEGF-C, designated siVEGF-C-1 and siVEGF-C-2. Following treatment with siRNA for 72 hours, both mRNA and protein were harvested for subsequent analyses. A, mRNA was reverse transcribed using random hexamer primers and the resulting cDNA was used for qPCR analysis. To calculate the abundance of VEGF-C mRNA, the value obtained using VEGF-C-specific primers was compared to that obtained using primers for 36B4. B, Conditioned media were collected and analyzed via ELISA to determine the amount of VEGF-C secreted from the cells. C, Immunoblot analysis comparing the expression of WDFY-1 and LAMP-2 in cells treated with siVEGF-C-1 or siVEGF-C-2.

Legends for Supplemental Figures

Supplemental Figure 3: Representative confocal microscopy images illustrating the effects of The VEGF-C/NRP-2 depletion on autophagosomal trafficking. PC3 cells stably expressing mCherry-GFP-LC3 were seeded onto coverslips. Following the depletion of either VEGF-C or NRP-2, cells were imaged using an LSM710 microscope with appropriate filters. Green and yellow punctae are indicative of autophagosome formation. Red-only structures correspond to autolysosomes as the lysosomal lumen is highly acidified and GFP is acid-labile. Ten representative images from each experimental group are provided.

Supplemental Figure 4: The effects of VEGF-A depletion on the level of WDFY-1 and LAMP-2 expression. To analyze whether VEGF-A was redundant for VEGF-C, we transfected PC3 cells with cocktail VEGF-A siRNAs or scrambled siRNA. Following 72 hours of transfection, mRNA and proitein were harvested from the cells. A, Immunoblot analysis of WDFY-1 and LAMP-2 following VEGF-A depletion. B, mRNA was reverse transcribed and the resulting cDNA was used for qPCR analysis comparing the expression of VEGF-A to 36B4 expression. C, To determine whther VEGF-A regulated autophagy, we performed autophagic flux assays, in which the degradation of LC3 was prevented by the addition of BAFM. Cells were treated with 5- or 10-nM does of docetaxel prior to BAFM treatment. D, Graphical representation of immunoblot data in C.

Legends for Supplemental Figures

Figure 5: The effects of VEGF-C/NRP-2 depletion or blockade on autophagic flux in other cancer cell lines. A, Du145 cells were transfected with VEGF-C siRNA and treated with either a 5- or 10-nM dose of docetaxel. Additionally a subset of each experimental group was treated with BAFM. Immunoblots for LC3 were then performed to determine whether there is a change in autophagic flux in these cells following the depletion of VEGF-C. B, Similarly, CaPan-1 cells were treated with either VEGF-C or NRP-2 siRNA and autophagic flux was analyzed via immunoblot. C, Alternatively, autophagic flux was analyzed in CaPan-1 cells treated with either a VEGF-C function-blocking antibody or a soluble form of NRP-2.

Supplemental Figure 6: Representative confocal images of cell viability in PC3 cells depleted of NRP-2. PC3 cells were seeded into chamber slides and treated with either scrambled or NRP-2 siRNAs. Following treatment with 20- or 50-nM doses of docetaxel, the cells were stained with Hoechst, PI and Yo-pro and imaged using an LSM710 microscope with appropriate filters. Five representative images of each experimental group are provided.

Supplemental Figure 7: Cell viability following treatment with NRP-2 siRNA for 48 hours. Immunofluorescent analysis of cell death in PC3 cells treated with NRP-2 siRNA and a 10-nM dose of docetaxel for 48 hours. Five representative images for each data set are provided.

Legends for Supplemental Figures

Supplemental Figure 8: Effect of treatment with BAFM on autophagy and cell survival. A, PC3 cells stably expressing mCherry-GFP-LC3 were treated with BAFM, docetaxel, or a combination of both agents. The progression on autophagy was then assayed via immunoflurescence. One representative image is provided for each experimental group. B, PC3 cells were seeded into chamber slides and following treatment with docetaxel, BAFM, or a combination of both, the cells were stained with Hoechst, PI and Yo-Pro. Immunofluorescent imaging was used to determine the amount of cell death caused by each treatment. Three representative images are provided.

Supplemental Figure 9: A. Representative images of VEGF-C-expressing LNCapC4-2B cells following treatment with siNRP-2, docetaxel, and BAFM. Following docetaxel treatment, cells were stained with Hoechst, Yo-Pro, and PI per manufacturer's instructions and cell death was analyzed via immunofluorescent microscopy. Three representative images for each group are provided.
B, Following the expression of VEGF-C, C4-2B cells were treated with 10-nM doses of docetaxel and BAFM. Cells were then harvested, lysed, and blotted for LC3. The amount of autophagic flux was then calculated as described previously. A graphical representation of this analysis are presented.

Legends for Supplemental Figures

Supplemental Figure 10: Representative images of VEGF-C-expressing LNCapC4-2 cells following treatment with siNRP-2, docetaxel, and BAFM. Following docetaxel treatment, cells were stained with Hoechst, Yo-Pro, and PI per manufacturer's instructions and cell death was analyzed via immunofluorescent microscopy. Three representative images for each group are provided.

Supplemental Fig 11. The role of the VEGF-C axis in cell survival regulation via autophagy. PC3 cells were depleted of VEGF-C for 48hrs before over-expressing a siRNA-resistant full-length VEGF-C clone for 24hrs. A cell death assay was then performed as described previously to determine whether the expression of siRNA-resistant VEGF-C could rescue cell viability.

Supplemental Figure 12: Representative confocal images of Du145 cells treated with VEGF-C siRNA and docetaxel. Du145 cells were depleted of VEGF-C and stained with Hoechst, YoPro, and PI in order to analyze cell death via immunofluorescence. Three representative images of each experimental group are provided.

Supplemental Figure 13: Representative confocal images of VEGF-C-depleted CaPan-1 cells treated with 10- and 20-nM doses of gemcitibine. CaPan-1 cells were treated with scrambled and VEGF-C siRNAs, and either a 10- or 20-nM dose of gemcitibine. Following staining with Hoechst, PI, or YoPro, cell death was visualized via confocal microscopy. Three representative images are provided.

Legends for Supplemental Figures

Supplemental Figure 14: Representative confocal images of NRP-2-depleted CaPan-1 cells treated with gemcitibine. CaPan-1 cells were treated with scrambled and NRP-2 siRNAs and a 30-nM dose of gemcitibine. Following staing with Hoechst, PI and YoPro, the cells were imaged using confocal microscopy. Five representive images are shown.

Supplemental Figure 15: The effects of function-blocking VEGF-C antibody/soluble NRP-2 on CaPan-1 cell survival in the presence of gemcitibine. Immunofluorescent determination of cell survival in CaPan-1 cells treated with gemcitibine and either a function-blocking antibody against VEGF-C (A) or soluble NRP-2 (B). Ten fields per experimental group were counted and the number of dead cells was divided by the total number of cells to determine the amount of dead/dying cells. Standard deviations were calculated for each data set and are included in the graphs. Additionally, a t-Test was performed to determine whether the results obtained in the experimental groups were statistically different from controls.

Supplemental Figure 16: Representative images of PC3 cells depleted of WDFY-1 and either VEGF-C or NRP-2. Immunofluorescent analysis of apoptosis in PC3 cells depleted of WDFY-1 alone or in concert with either VEGF-C or NRP-2. Three representative images are provided.

Legends for Supplemental Figures

Supplemental Figure 17: (A) Immmunoblot results showing pS6K, pAKT, LC3-II , total AKT and total S6K levels in LNCaP C4-2 cells treated with or without recombinant VEGF-C protein (100 nM) and docetaxel (15 nM). Docetaxel was added after 8 hours of VEGF-C addition for another 20 hours. (B) Immunoblot results showing expression of pS6K and pAKT in VEGF-C expressing stable clone of LNCaP C4-2 and vector only stable clone of LNCaP C4-2. Cells were treated with different doses of docetaxel for 20 hours. (C) Determination of the role of Akt in VEGF-C-dependent autophagic activation. Following the depletion of VEGF-C, the activation of the mTOR pathway was determined by monitoring the level of S6K phosphorylation. Akt was then depleted using 5- and 20-nM doses of Akt siRNA to determine its effect on mTOR activation.