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# Over-Expression of Ly6/Plaur Domain Containing 6b (Lypd6b) in Ovarian Cancer

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## Abstract

Advanced stage serous ovarian cancer is metastatic disease with poor prognosis requiring the identification of new therapeutic and prognostic targets. We examined the gene expression of 20 advanced stage serous ovarian cancers compared to eight cases of normal ovarian surface epithelium using Affymetrix human genome U133 Plus2.0 GeneChip® array and identified the over-expression of LY6/PLAUR domain containing 6B (LYPD6B) in cancers. The function of LYPD6B is unknown, however, the LYPD6B sequence encodes an amino acid region of high similarity to snake venom toxins and the PLAUR domain, a domain present in genes involved in regulating invasion and metastasis. We identified three LYPD6B mRNA variants and termed them as LYPD6B_a, LYPD6B_b and LYPD6B_c. We found that the variant LYPD6B_a is predominantly expressed in late stage serous ovarian cancers by quantitative real time PCR. All three variants of the recombinant V5-tag LYPD6B proteins are expressed on the cell membrane of OVCAR3 ovarian cancer cells. Four different shRNAs were used in knockdown of mRNA and protein of LYPD6B in OVCAR3 cells. However, these LYPD6B knockdown cells did not show any change of cell morphology, cell proliferation and cell migration. We noted a dramatic over-expression of several other LY6/PLAUR domain containing transcripts in ovarian cancer. Of these, LYPD1 expression is higher than that of LYPD6B. In summary we identified the high expression of LYPD6B and LYPD1 in ovarian cancers and that these may encode proteins useful in diagnostic or prognostic purposes or for the evaluation of recurrence in ovarian cancer.

**Keywords:** Serous ovarian cancer; Gene expression analysis; LY6/PLAUR domain containing 6B (LYPD6B)

## Introduction

Significant efforts are currently underway to identify protein and RNA biomarkers relevant to early detection of epithelial ovarian cancer. These efforts are justified by survival data from women with ovarian cancer which strongly suggest that women with stage I cancers have a 5 year survival rate greater than 75% [1]. However, most cases are initially diagnosed with advanced stage cancer and have an extremely poor 5 year survival rate of less than 25% [2]. Various factors may contribute to the favorable outcome in early diagnosed cases. The biologic differences between early (stage I) and advanced (stage III/IV) cancers are poorly defined, and as are the factors present in recurring early stage cancers.

The conventional view is that stage I cancers will eventually progress to advanced disease given time, but little evidence supports this assumption. Whereas every stage III/IV cancer must at some point be a stage I cancer by convention, the length of time that aggressive cancers remain confined to the ovary or disseminate from fallopian tube precursor lesions is unknown. Data suggest that significant biologic differences exist between early and late stage cancers. Specifically, the majority of initially diagnosed stage III cancers are of serous histotype. In contrast, only about 25% of stage I cancers are of serous histotype with roughly equivalent percentages of endometrioid, mucinous and clear cell types [3]. Furthermore, early stage serous cancers have similar expression patterns to advanced stage cases with good prognosis [3]. These data suggest that a class of highly aggressive serous cancers form the majority of lethal disease and that most of these cases are not diagnosed early. In addition, even though serous cancers are the predominant histotype, a large early cancer detection trial using ultrasound and CA125, detected few of these as stage I ovarian cancers [4]. These findings suggest that the majority of aggressive serous cancers may progress quickly through stage I disease whereas some early stage cancers may remain relatively indolent and confined to the ovary for extended periods of time. Understanding the underlying biology of these differences and defining the characteristics of more aggressive cancers is critical to developing more effective therapies and prognostics.

We performed Affymetrix expression arrays in order to specifically identify those transcripts up-regulated in a group of advanced serous ovarian cancers and found over-expression of a poorly characterized transcript detected by probe 228360_at in late stage serous ovarian cancers. The transcript encodes a protein with a LY6/PLAUR domain and is designated as LYPD6B based on this homology by the NCBI. Furthermore, several transcripts of this locus were cloned and reported as LYPD7 in 2008, however, the gene function is unknown and there are no reports of this gene associated with human disease [5]. Three mRNA variants of LYPD6B (gene accession No. NM_177964, BC018203 and AF435957) are represented in gene databases and these transcripts all contain an amino acid region of high similarity to snake venom toxins and the PLAUR domain, a domain present in genes involved in regulating invasion and metastasis. The best characterized protein with this domain is the cell surface receptor for urokinase plasminogen activator, PLAUR, more commonly known as uPAR. uPAR is an extensively studied peptide and its role in cancer recently reviewed [6]. It is clearly involved in modulation of the extracellular space; its proteins and how these interactions influence invasion or metastasis were reviewed. uPAR is also up-regulated in cancers, particularly those with propensity for metastasis, poor prognosis and invasion [6] and is also known to be necessary for cancer growth and maintenance as

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well as to invasion [7]. In addition to LYPD6B, we noted up-regulation of several other LY6/PLAUR domain containing transcripts from the gene array data.

In this study, we describe the expression of LYPD6B and its variants in ovarian cancer. We also identify co regulated genes including LYPD1 another LY6/PLAUR domain containing gene also highly expressed in ovarian cancer. Furthermore we perform some initial characterization on the cellular localization and functional consequences of LYPD6B mRNA knockdown in ovarian cancer cells.

## Materials and Methods

### Ovarian tumor samples and cell lines

Surgical specimens of twenty human ovarian cancer and eight normal ovarian tissues were obtained from the Southeast division of the Cooperative Human Tissue Network (CHTN). Normal ovary samples used in initial array studies were enriched for surface epithelium by rolling the wooden end of a sterile Q-tip over the surface and subsequently dispersing adherent cells in ice cold saline. Cells were collected following centrifugation and immediately lysed in TRIzol. The study was approved as exempted by institutional review board.

Ovarian cancer cell line OVCAR3 was obtained from the National Cancer Institute NCI 60 repository, Frederick MD. Ovarian clear cell carcinoma cell lines ES-2, TOV21G and normal ovarian surface epithelium NOV-31 were obtained from Dr. Anne-Marie Mes-Masson (Université de Montréal, Québec, Canada). Cells were maintained in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 mg/ml of streptomycin, 100 units/ml of penicillin and 1µg/ml of Fungizone) at 37°C in 5% $CO_2$.

### Expression array

Tissue samples were subjected to RNA isolation using TRIzol (Invitrogen, Carlsbad, CA, USA) and an additional purification using the RNeasy Kit (Qiagen, Valencia, CA, USA) following the recommendation of the manufacturer. Following isolation of RNA, the integrity of each RNA sample was verified and amplified cRNA was labeled, hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChip® expression arrays, washed according to the manufacturer's specifications (Affymetrix, Inc, Santa Clara, CA, USA) and scanned using GeneChip scanner 3000.

Expression Console software (Affymetrix Inc.) was used to determine the signal values by MAS5 algorithm. Total intensity normalization was applied by normalizing all arrays to trimmed mean value of 500. All the statistical calculations were done on logarithmic values of signals to the base 2. The global expression profiles of tumors were found significantly different from normal tissues at global test $p < 10^{-7}$ (BRB ArrayTools software ver. 4) [8]. Differentially expressed genes between tumors and normal tissues were determined by two-sample T-tests at two-tailed $p$-value < 0.001. These were further screened by selecting the genes called present in 95% or more tumors and absent in all normal tissues where the present and absent calls are based on MAS5 detection $p$-value (of Wilcoxon signed rank test) < 0.06 and > 0.06 respectively. There were 40 unique genes satisfying these criteria and up-regulated in cancers by 30-fold than normal tissues.

### Reverse transcription and polymerase chain reaction

Total RNA was extracted from tissues and cultured cells by direct homogenization in TRIzol (Invitrogen) and spectrophotometry was used for quantification. Total RNA was treated with DNase I (Invitrogen) and reverse transcription was performed using SuperScript® III reverse transcriptase (Invitrogen) according to the manufacturer's instruction. PCR was performed using GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Carlsbad, CA, USA). Quantitative real time RT-PCR analysis was performed using Mx3000P (Agilent Technologies, Santa Clara, CA, USA) thermal cycler.

### Quantitative real time PCR (qPCR)

All three LYPD6B variants were detectable by Hs00962009_m1 TaqMan probe set from Applied Biosystems (Foster City, CA, USA). TaqMan MGB probe and primers specific for the three variants of LYPD6B were designed using the software Primer Express 1.5 (Applied Biosystems). Primers and MGB probe for LYPD6B are listed in Table 2. qPCR was performed at 95°C for 2 min followed by 40 cycles of 95°C for 2 sec and 60°C for 30 sec, using PerfeCTa qPCR FastMix (Quanta BioSciences, Gaithersburg, MD, USA) according to the manufacturer's instruction. Each PCR product was subcloned into the TA cloning plasmid pCR4-TOPO vector (Invitrogen) and used as a positive control for qPCR analyses. The number of molecules of specific gene products in each sample was determined using a standard curve generated by amplification of $10^2$–$10^8$ copies of the control plasmid.

### N-V5 tag recombinant LYPD6B protein

Cloning of LYPD6B variants was performed using the Gateway system (Invitrogen). In brief, primers specific for the complete coding region of LYPD6B_a (NM_177964), LYPD6B_b (BC018203) and LYPD6B_c (AF435957) variants were designed using the computer program OLIGO 4.0 (National Biosciences, MD, USA) and were based on published sequences in GenBank. LYPD6B_a; forward 5' - caccatgttgctgattactctgagtgc -3', reverse 5'- taggaatggt ggcatcacag -3'. LYPD6B_b; forward 5' - caccatgctgctcctctgtcacg -3', reverse 5' – tcacggcaaacactgcatagt – 3'. LYPD6B_c; forward 5' - caccatgttatataagagttcggaccgc -3', reverse 5'- taggaatggtggcatcacag -3'.

Total RNA obtained from OVCAR 3 cells was used to prepare cDNA using SuperScript® III reverse transcriptase and random hexamers as directed by the manufacturer. Specific variants were cloned following PCR under the cycling conditions of 95°C for 2 min followed by 35 cycles of 95°C for 5 sec, 60°C for 30 sec, 72°C for 1 min, using AccuPrime™ *Pfx* DNA Polymerase (Invitrogen). Each PCR product of LYPD6B variants was cloned in pENTR / D-TOPO plasmids. Clones for all three LYPD6B variants open reading flame were confirmed by DNA sequencing. These resulting entry clones were used for Gateway LR clonase II (Invitrogen) directed recombination in the Gateway expression vector pcDNA3.1 / nV5-DEST (Invitrogen) which was designed to add an N-terminal V5 tag. The resulting plasmids (pcDNA3.1 / nV5-LYPD6B variants a, b and c) were transfected into the OVCAR 3 cell line using FuGENE (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Membrane and cytosolic protein fractions of transformed OVCAR 3 cells were prepared using the Protein Extract Native membrane protein extraction kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instruction.

### Knockdown of LYPD6B by shRNA

Non-Target control and LYPD6B-specific small hairpin RNAs lentiviral particles were purchased from Sigma (Boston, MA, USA). OVCAR 3 was transduced with four different shLYPD6B-lentivirus (LYPD6B #1; TRCN0000162314, LYPD6B #3; TRCN0000164602, LYPD6B #4; TRCN0000164910, LYPD6B #5; TRCN0000166404) or Non-Target shRNA control lentivirus (SHC002V) and selected for 1 week in 2µg/mL puromycin and used for total RNA isolation, western blot analysis and in proliferation assays.

## Western blot analysis

Cells were directly lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) with Halt Protease Inhibitor Cocktail (Thermo Scientific) and cellular protein concentrations were determined by DC protein assay (Sigma). SDS-PAGE and western blotting were performed using the NuPAGE electrophoresis system (Invitrogen) according to the manufacturer's instruction. In brief, equal amounts of proteins were boiled in NuPAGE sample reducing agent and LDS sample buffer (Invitrogen) and subjected to NuPAGE 4-12% Bis-Tris gel electrophoresis separation. Subsequently, the gels were transferred onto a polyvinylidene difluoride membrane and nonspecific binding was blocked by immersing the membrane in 5% nonfat milk for 1 hour at room temperature. Immunoblots for nV5-LYPD6B was preformed as follows. Membranes were incubated with horseradish peroxidase-conjugated (HRP) rabbit anti-V5 tag antibody (Invitrogen) at 1: 5000 dilutions for one hour at room temperature. The membrane was then washed with PBS plus 0.05% Tween-20 (PBS-T) for one hour and detected by Amersham ECL advance western blotting detection kit (GE healthcare, Little Chalfont, Buckinghamshire, UK).

Immunoblot for LYPD6B was preformed as follows. Anti-human LYPD6B rabbit antiserum was prepared by Covance (Princeton, NJ, USA). Antiserum was generated against KLH conjugated CKHHSRDSEHTE peptide of LYPD6B. This peptide amino acid sequence is located spanning exon 8 to exon 9 of all isoforms of LYPD6B protein. Therefore, this antiserum may detect all of the variants of LYPD6B. Anti-human LYPD6B rabbit antiserum was used for primary antibody of immunoblotting and HRP-anti rabbit IgG (GE Healthcare) at 1:20000 dilutions was used for secondary antibody at room temperature for one hour. The membrane was then washed with PBS-T as described and the signal detected by ECL.

## Proliferation assay

Following different shRNA transductions, the cells were trypsinized and counted with hemocytometer. Proliferation assays were performed as follows. Cells were seeded in plastic 96 well plates at a density of 2 x $10^3$cells per well and cultured over-night with media containing 100µl of 10% FBS / DMEM F12. Numbers of viable cells were measured on day 5 according to the manufacturer's instructions by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) that measures the reduction of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt compound (MTS) by living cells only. Briefly, 20µl of MTS is added to each well incubated at 37°C / 5% $CO_2$ in the dark for one hour. The absorbance at 490nm was recorded by using a micro plate reader. Each cell type was seeded in triplicate and experiments were repeated three times.

# Results and Discussion

## Identification of LYPD6B in ovarian cancer samples

We performed gene expression analysis on a set of advanced stage serous ovarian cancers and normal ovary enriched for ovary surface epithelium. We identified genes with characteristics of absent expression in normal tissues and high expression (present call) in at least 95% of the cancers. We identified known and established ovarian cancer markers in this screen including MUC16 [9,10], PAX8 [11,12,13], FOLR1 [14] and Claudin 4 [15,16]. MUC16 is well established as a prognostic marker and used clinically for recurrence evaluation and Folate Receptor is currently being evaluated as a therapeutic target [17,18]. These findings suggest the approach we utilized has significant merit and that other transcripts with similar expression patterns might represent additional proteins of relevance to ovarian cancer biology and be useful for therapeutic, diagnostic and/or prognostic purposes (Table 1). We also identified a variety of known and novel transcripts with very similar expression characteristics (Figure 1). However, we focused our efforts on the unknown genes. Affymetrix probeset 228360_at represented one of the unknown transcripts with absent expression in all normal samples and high expression in all cancer samples. The transcript was upregulated 31 fold compared to the normal ovary surface epithelium and the transcript contains an open reading frame of high similarity to snake venom toxins and the PLAUR domain (Figure 2). At the time we performed the array the gene was not named. Subsequently it has been named LYPD6B based its LY/PLAUR domain homology and similarity to other LYPD family members and LYPD7 by another publication [5].

We examined the expression of other LY6/PLAUR domain containing transcripts in our ovarian cancer dataset and for which Affymetrix probes targeted them. We noted several members that were highly expressed in ovarian cancer including LYPD1 which contained robust expression and which exhibited the highest correlation with LYPD6B (Figure 3). Yu et al. [19] reported LYPD1 is upregulated in HeLaHF cells. HeLaHF is a non-transformed variant (flat and non-refractile morphology, markedly decreased growth in soft-agar, and loss of in vivo tumor growth potential) of HeLa cells isolated following exposure to the mutagen ethylmethane sulfonate. The knockdown of LYPD1 mRNA in HeLaHF cells by RNAi resulted in an 18-fold increase in anchorage-independent growth compared to control HeLaHF cells. Over-expression of recombinant LYPD1 induced apoptosis and

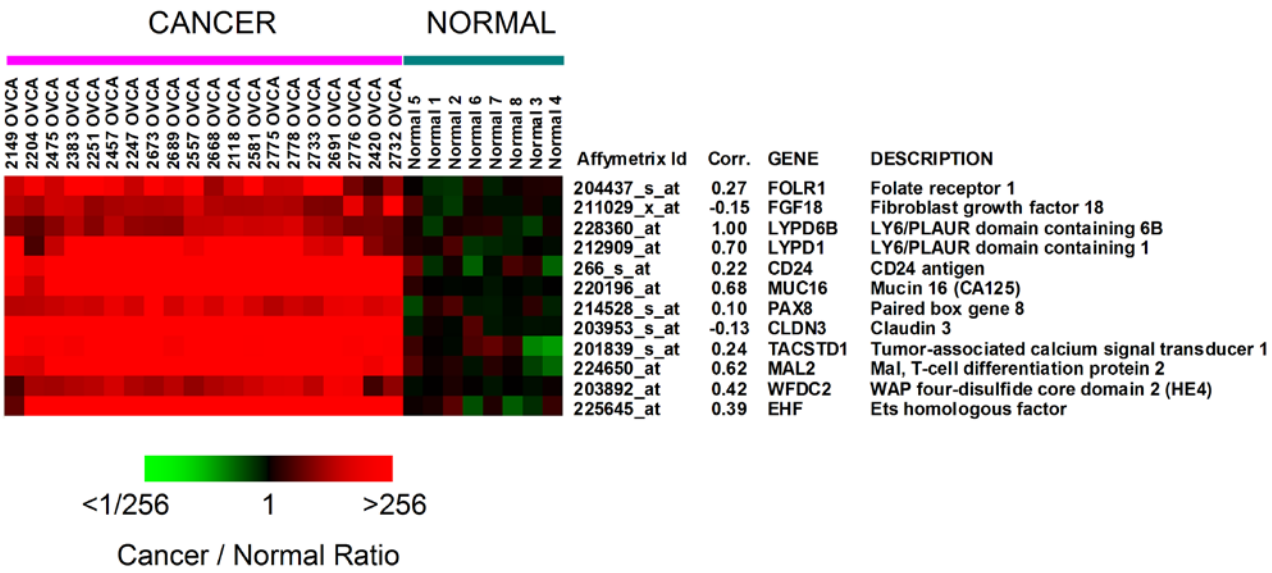
| | Transcript /Gene | *Expression Ave. Tumor | *Expression Ave. Normal | Fold | p-value Tu vs. Nor | Reference |
|---|---|---|---|---|---|---|
| A. | MUC16 (CA125) | 7539 | 6 | 1303 | 8.8×10-16 | [9,10] |
| | CLAUDIN 4 | 1570 | 9 | 170 | 1.8×10-19 | [15,16] |
| | PAX8 | 1695 | 15 | 110 | 2.6×10-14 | [11-13] |
| | FOLR1 | 5281 | 44 | 120 | 1.8×10-9 | [14] |
| B. | LYPD6B | 1135 | 36 | 31 | 1.1×10-9 | [5] |

*Expression based on signal count geometrically averaged from 20 stage IIIC/IV serous ovary cancers and from 8 normal ovaries. All normal ovaries were also scored as absent expression using Affymetrix absent /present call

**Table 1:** General characteristics of known and newly identified ovary cancer transcript up-regulated in stage IIIC/IV serous ovarian cancers.

| LYPD6B variant | Accession | Forward primer (5' to 3') | Location | Reverse primer (5' to 3') | Location | MGB prove (5' to 3') | Location |
|---|---|---|---|---|---|---|---|
| a | NM_177964 | tgcaaacctttcactgttcca | exon 5 | gagagcgtgacagaggagcag | exon 6 | attctccttctcaagatataag | exon 5 to 6 |
| b | BC018203 | cgggcggcgagatatg | exon 1 to 4 | gcggtccgaactcttatataacatc | exon 4 to 6 | ctgctcttgttaatcacat | exon 4 |
| c | AF435957 | caggacttcagagtgtttgttttcag | exon 2 | gcagaagtgtggcatatctgga | exon 3 to 4 | tgaagattatcgcggaacac | exon 2 to 3 |

**Table 2:** Real-time PCR primers and MGB probe sequence for LYPD6B variants.




| Affymetrix Id | Corr. | GENE | DESCRIPTION |
|---|---|---|---|
| 204437_s_at | 0.27 | FOLR1 | Folate receptor 1 |
| 211029_x_at | -0.15 | FGF18 | Fibroblast growth factor 18 |
| 228360_at | 1.00 | LYPD6B | LY6/PLAUR domain containing 6B |
| 212909_at | 0.70 | LYPD1 | LY6/PLAUR domain containing 1 |
| 266_s_at | 0.22 | CD24 | CD24 antigen |
| 220196_at | 0.68 | MUC16 | Mucin 16 (CA125) |
| 214528_s_at | 0.10 | PAX8 | Paired box gene 8 |
| 203953_s_at | -0.13 | CLDN3 | Claudin 3 |
| 201839_s_at | 0.24 | TACSTD1 | Tumor-associated calcium signal transducer 1 |
| 224650_at | 0.62 | MAL2 | Mal, T-cell differentiation protein 2 |
| 203892_at | 0.42 | WFDC2 | WAP four-disulfide core domain 2 (HE4) |
| 225645_at | 0.39 | EHF | Ets homologous factor |

**Figure 1: Up-regulated transcripts in ovarian cancers.** Heat map demonstrating the expressions of selected genes including *LYPD1* and *LYPD6B* that are highly up-regulated in ovarian cancers compared to the average normal tissue level determined by using Affymetrix HG-U133 Plus 2.0 GeneChip® arrays. The correlation coefficients to LYPD6B profile within cancers alone are indicated by Corr. Well established ovarian cancer markers as well as other known genes which match a profile of ubiquitous high expression in cancer compared to absence in normals are depicted.

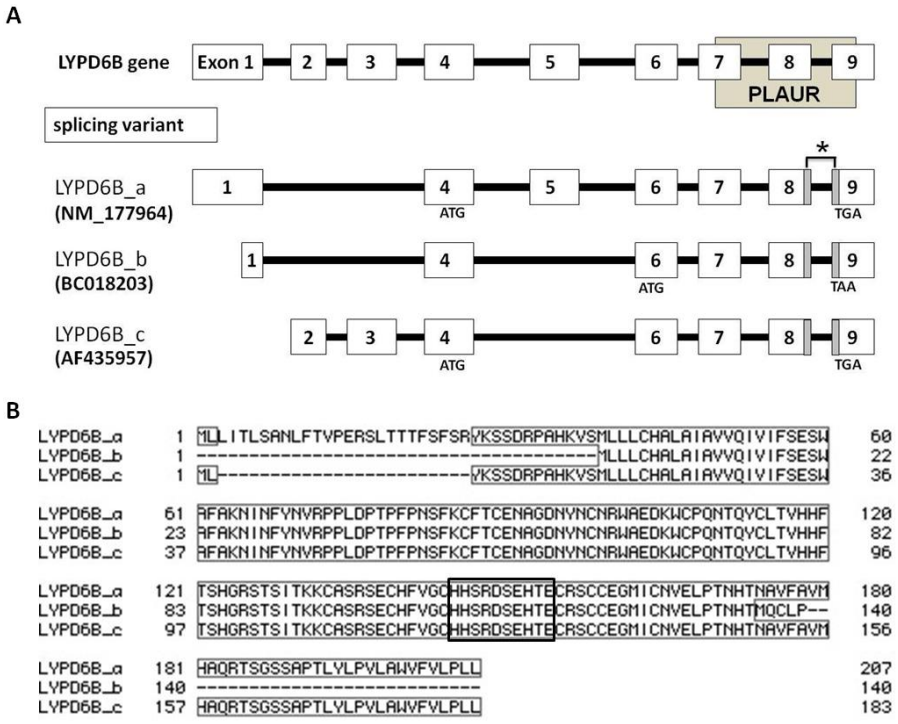



**Figure 2: Genomic organization, mRNA splicing variants and protein sequences of human LYPD6B.** The *LYPD6B* gene consists of 9 exons. Exon 7 to 9 contains the Ly-6 antigen/uPA receptor-like domain (PLAUR domain) and this domain is conserved in all splicing variants. LYPD6B_b variant utilizes different start and stop codons at exon 6 and exon 9 respectively. The LYPD6B_c variant form differs by the splicing out of exon 5. The asterisk shows the mRNA and genomic context localization of the HHSRDSEHTE peptide sequence used for the generation of LYPD6B antibody. (B) Amino acid sequences of LYPD6B isoforms. The boxed amino acid sequences represent the antigen peptide sequence used for LYPD6B antibody.

resulted in reduction of anchorage-independent growth in soft agar in HeLa cells. Yu et al. concluded that LYPD1 suppresses cell growth by triggering apoptosis. The function of LYPD1 in ovarian cancer is unknown, however, this is the first report of over-expression LYPD1 in cancer. Interestingly LYPD6 which is aligned head to tail adjacent to LYPD6B on chromosome 6 exhibited relatively low expression as compared to LYPD6B, its most highly related family member.

We examined the expression of LYPD6B in other publically available ovarian cancer datasets which also indicated high levels of expression in cancers. In a dataset containing 53 advanced serous cancers and 10 normal tissues LYPD6B was up-regulated 7.5-fold at $p = 9.5\times10^{-12}$ in cancers [20] (Figure 4). Further examination of an even more extensive dataset including endometrioid, clear cell and borderline cases substantiated the up-regulation of LYPD6B in neoplastic ovarian diseases (data not shown) [21].

## Expression levels of LYPD6B transcript variants

Three splicing variants for LYPD6B are represented in gene databases, we termed LYPD6B_a (NM_177964), LYPD6B_b (BC018203) and LYPD6B_c (AF435957, see Evidence Viewer, http://www.ncbi.nlm.nih.gov/sutils/evv.cgi?taxid=9606&contig=NT_005403.17&gene=LYPD6B&lid=130576). These three variants all are predicted to encode differing peptides principally in the N-terminus. The Ly-6 antigen/uPA receptor-like domain (PLAUR domain) present in exon 7 to 9 remained constant in all variants. However, each of the variants also differs in the amino acid sequence of N and C-terminal regions detailed in Figure 2. We designed specific qPCR primer and probe sets for each LYPD6B variant (Table 2). We examined the expression of these variants in a tissue sample set that is different from the tissues

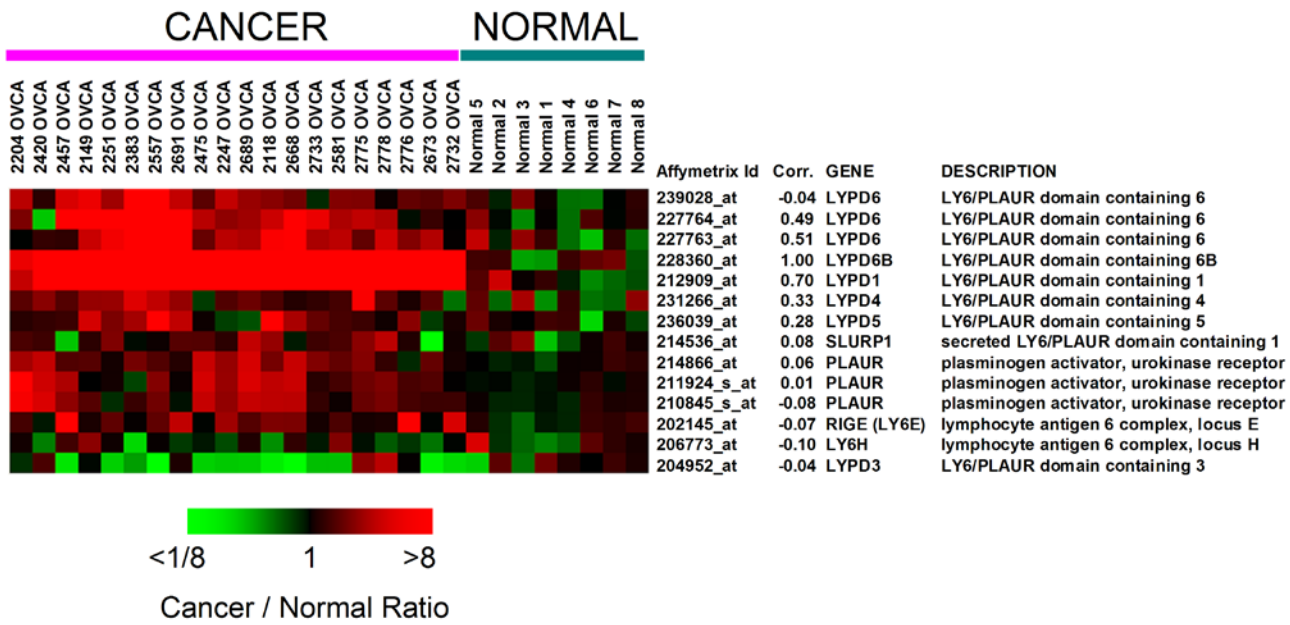



**Figure 3**: **Expression of PLAUR domain containing transcripts in ovarian cancers.** Relative expressions of LYPD family members, *PLAUR*, *RIGE*, and *LY6H* genes and other PLAUR domain containing transcripts in 20 stage IIIC/IV serous ovarian cancers and 8 normal ovary tissues determined by using Affymetrix HG-U133 Plus2.0 GeneChip® arrays. Expressions shown are relative to average of normal tissues. The correlation coefficients to LYPD6B profile within cancers alone are indicated by Corr.

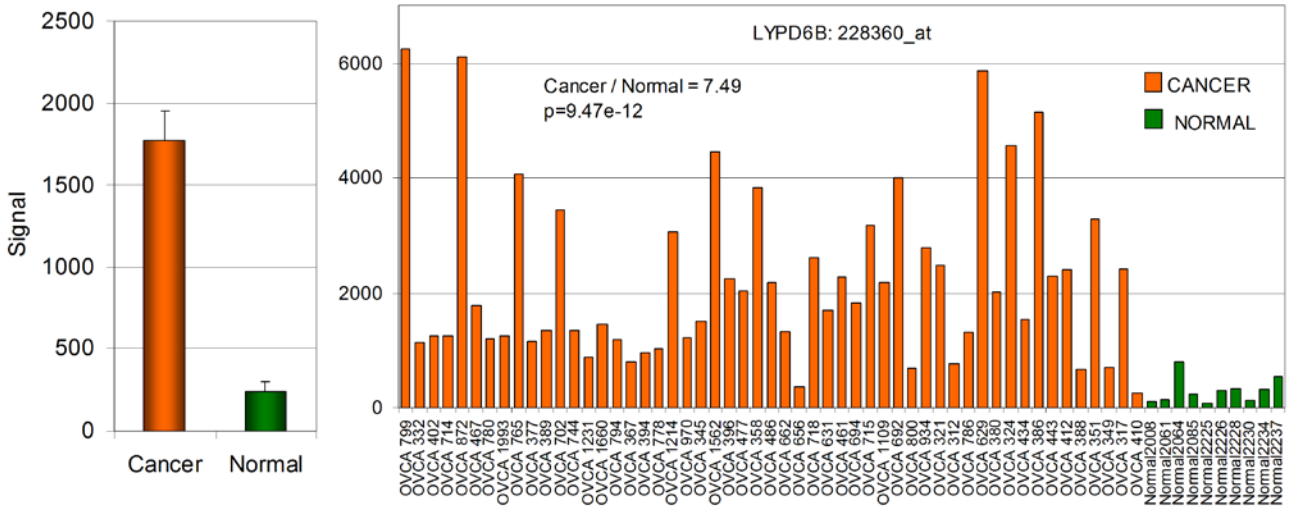



**Figure 4: Expression of LYPD6B in an independent dataset of ovarian cancer and normal samples.** Average expression levels of LYPD6B in Mok's data set of 53 advanced serous cancers vs. 10 normal tissues indicated about 7.5-fold up-regulation (*$p = 9.5\times10^{-12}$).

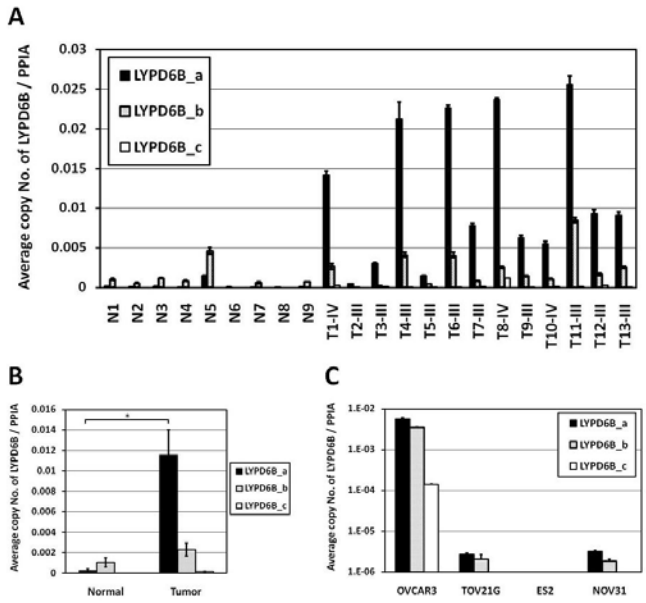



**Figure 5: Expression of LYPD6B mRNA variants in ovarian tissues.** (A) Expression levels of the LYPD6B variants in normal ovaries (n=9) and serous ovarian cancers (n=13) using quantitative PCR assays specific for each variant (Roman numerals designate stage of cancer). (B) Mean value of LYPD6B variants expression in normals and cancers. LYPD6B_a was 49 fold over-expressed in serous ovarian cancers compared to normal ovaries (SD; *$p$<0.001). (C) LYPD6B variant expression levels in several ovarian cancer cell lines. LYPD6B_a and b variants were highly expressed in OVCAR 3 cells. OVCAR 3 (Serous ovarian cancer), TOV21G (clear cell ovarian cancer), ES2 (clear cell ovarian cancer) and NOV-31 (normal ovarian surface epithelium).

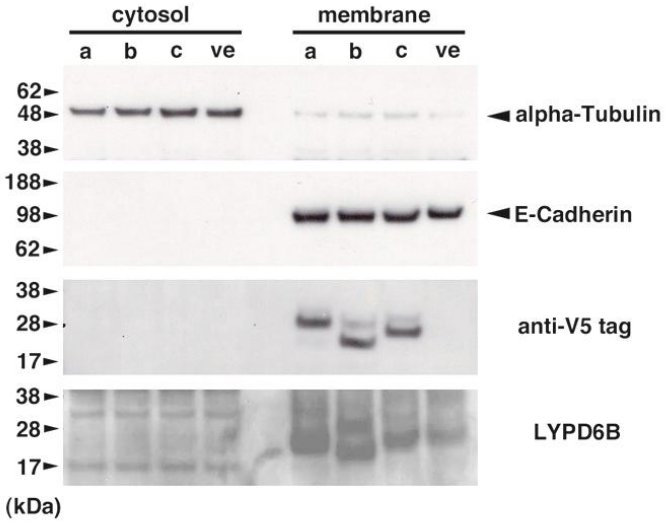



**Figure 6: Immunoblot analysis of LYPD6B.** N-terminal V5 tagged isoforms of LYPD6B were expressed in OVCAR 3 cells. Cytosol and membrane protein fractions were extracted from transfectants and those proteins were analyzed by immunoblots. Lane 1, alpha-tubulin (MW=55 kDa) was used for cytosol specific marker. Lane 2, E-cadherin (MW=120 kDa) was used for membrane specific marker. Lane 3, anti-V5 tag antibody clearly detected V5-tag LYPD6B isofoms. Deduced MW of V5-tag LYPD6B_a, b and c were 28.1 kDa, 23.8k Da and 25.4 kDa, respectively. Lane 4, immunoblot result of ant-LYPD6B antibody. Deduced MW of LYPD6B_a, b and c were 23.3 kDa, 15.9 kDa and 20.6 kDa, respectively.

utilized for array (9 normal ovarian samples and 13 stage IIIC or IV serous ovarian cancers) using quantitative PCR assays. Data from these assays show that LYPD6B_a is significantly over-expressed in serous ovarian cancers compared to normal ovarian samples (49 fold, $p$<0.001). Whereas, variants (b and c) exhibited expression levels that

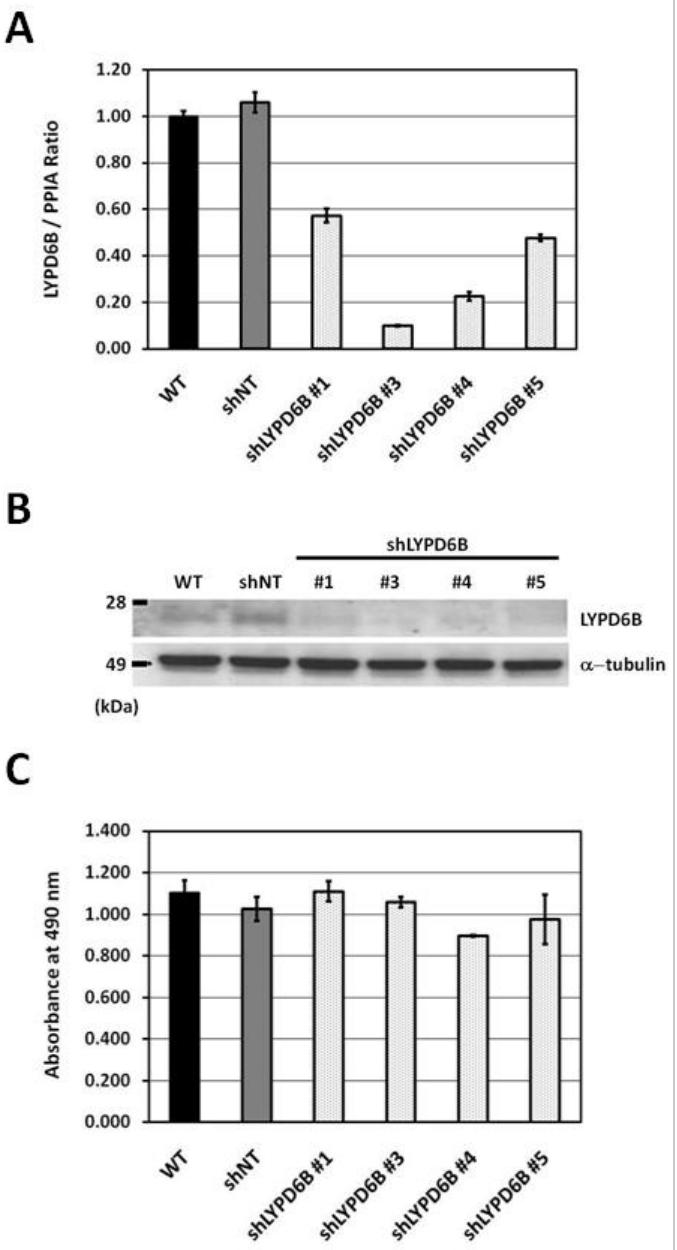



**Figure 7: shRNA mediated knockdown of LYPD6B.** Four different lentiviral shRNA particles of LYPD6B were transduced to OVCAR 3 cell. (A) qPCR was utilized to quantitate levels of LYPD6B in knockdown cells using assay Hs00962009_m1 that detects all variants. Knockdown efficiency of shLYPD6B #1, #3, #4 and #5 was 43, 90, 78 and 52 % compared to the parental cell LYPD6B expression levels, respectively. (B) LYPD6B immunoblot result of knockdown cell. Four different LYPD6B knockdown cells were lysed by RIPA buffer and equal amount of whole cell proteins were analyzed by immunoblot using anti-LYPD6B antibody. LYPD6B protein levels were significantly decreased in knockdown cells compared to the parental (WT) and non-target shRNA transducted (shNT) cell. (C) Proliferation assay of LYPD6B knockdown cell. 2000 cell of WT, shNT and four shLYPD6B were seeded to 96 well p late and cultured for 5 days and viable cells were measured by MTS assay. There were no differences of cell proliferation in these LYPD6B knockdown cells.

| Affymetrix Id | GENE | Description | R[1] | Signal[2] |
|---|---|---|---|---|
| 228360_at | LYPD6B | LY6/PLAUR domain containing 6B | 1.00 | 1135 |
| 229927_at | LEMD1 | LEM domain containing 1 | 0.87 | 681 |
| 1554576_a_at | ETV4 | ets variant 4 | 0.84 | 563 |
| 239619_at | ZNF395 | zinc finger protein 395 | 0.84 | 285 |
| 240261_at | TOM1L1 | target of myb1 (chicken)-like 1 | 0.81 | 270 |
| 235728_at | ZFP3 | zinc finger protein 3 homolog (mouse) | 0.80 | 379 |
| 241954_at | FDFT1 | Farnesyl-diphosphate farnesyltransferase 1 | 0.80 | 400 |
| 227452_at | LOC10049946 | hypothetical LOC100499467 | 0.79 | 516 |
| 212728_at | DLG3 | discs, large homolog 3 (Drosophila) | 0.79 | 474 |
| 203585_at | ZNF185 | zinc finger protein 185 (LIM domain) | 0.79 | 1540 |
| 224599_at | CGGBP1 | CGG triplet repeat binding protein 1 | 0.78 | 3545 |
| 231726_at | PCDHB14 | protocadherin beta 14 | 0.78 | 262 |
| 235513_at | ESTs | Transcribed locus | 0.78 | 219 |
| 204485_s_at | TOM1L1 | target of myb1 (chicken)-like 1 | 0.77 | 1346 |
| 219214_s_at | NT5C | 5', 3'-nucleotidase, cytosolic | 0.77 | 386 |
| 242354_at | ESTs | Transcribed locus | 0.77 | 594 |
| 209529_at | PPAP2C | phosphatidic acid phosphatase type 2C | 0.76 | 698 |
| 235148_at | KRTCAP3 | keratinocyte associated protein 3 | 0.76 | 949 |
| 240166_x_at | RG9MTD3 | RNAmethyltransferase domain containing 3 | 0.76 | 321 |
| 204990_s_at | ITGB4 | integrin, beta 4 | 0.76 | 1112 |
| 1560089_at | LOC286208 | hypothetical LOC286208 | 0.75 | 488 |
| 202172_at | VEZF1 | vascular endothelial zinc finger 1 | 0.75 | 2071 |
| 212729_at | DLG3 | discs, large homolog 3 (Drosophila) | 0.75 | 619 |
| 228638_at | FAM76A | Family with sequence similarity 76, member A | -0.75 | 624 |
| 200700_s_at | KDELR2 | KDELendoplasmic reticulum protein retention receptor 2 | -0.76 | 4590 |
| 225992_at | MLLT10 | myeloid leukemia translocated to, 10 | -0.76 | 1256 |
| 202838_at | FUCA1 | fucosidase, alpha-L- 1, tissue | -0.77 | 1930 |
| 218799_at | GPN2 | GPN-loop GTPase 2 | -0.77 | 466 |
| 214583_at | RSC1A1 | regulatory solute carrier protein, family 1, member 1 | -0.77 | 251 |
| 201212_at | LGMN | legumain | -0.85 | 1698 |
| 218927_s_at | CHST12 | carbohydrate (chondroitin 4) sulfotransferase 12 | -0.90 | 636 |

[1]Correlation coefficient to LYPD6B; [2]Geometric average signal of cancers

**Table 3:** Transcripts correlated with LYPD6B in 20 stage IIIC/IV serous ovarian cancers of data set (1).

were similar in normal and cancer tissues (Figure 5A-B). Moreover, we examined expression levels of these variants in a panel of ovarian cancer cell lines. LYPD6B_a and LYPD6B_b were highly expressed in serous ovarian cancer cell line OVCAR 3, LYPD6B expressions in TOV21G (clear cell carcinoma), ES2 (clear cell carcinoma) and NOV-31 (normal ovarian surface epithelium) were very low or under detection levels (Figure 5C). These results suggest that LYPD6B_a is the variant over-expressed in serous ovarian cancer.

## Localization of LYPD6B protein

A previous study detailed the cloning of some of the splice variants of LYPD6B_a (termed LYPD7 in that paper) in mouse and human [5]. This study also ectopically expressed a Myc tagged version of LYPD6B_a in HeLa cells a cell type not normally expressing the message for LYPD6B. In their experiments the Myc tagged version of LYPD6B_a demonstrated a diffuse intracellular expression. These data were quite surprising as protein homology and Pfam similarity of the LYPD6B isoforms suggest that they will be GPI anchored cell surface molecules similar to other LY6 family members. We examined the expression of N terminal V5 tagged versions of all three LYPD6B predominant isoforms in OVCAR 3 ovarian cancer cells, cells normally expressing LYPD6B message (Figure 5C). We isolated membrane and cytosolic fractions and performed immunoblotting of OVCAR 3 expressing V5 tagged versions of LYPD6B. As expected from protein homology predictions LYPD6B was exclusively present on the cell membrane components (Figure 6). Controls of alpha-tubulin and E-Cadherin for cytosolic and membrane fractions demonstrated the purity of our cell fractions. Furthermore, we developed antibody reagents specific for LYPD6B. KLH conjugated CKHHSRDSEHTE peptide of LYPD6B located within exon 8 to exon 9 of all LYPD6B isoforms protein was used to make antibodies in rabbit (Figure 2). Immunoblotting results show that this antibody detects an approximately 23 kDa protein in the membrane fraction of pcDNA3.1 V5-DEST control plasmid transfected OVCAR 3 cells (Figure 6). In non transfected OVCAR 3 the antibody detected a band at approximately 23 kDa. Consistent with the predicted molecular weight of dominantly expressed the LYPD6B_a isoform (Figure 6). Furthermore this band was greatly diminished in LYPD6B knockdown cells (see below).

## Knockdown of LYPD6B

Using pathway-specific reporter gene assays, Ni J et al. [5] showed that LYPD6B (termed LYPD7 by them) upregulated expression of an AP1 (Phorbol myristate acetate) reporter gene in a dose-dependent manner. The AP-1 transcription factor a dimer of Jun, Fos and ATF proteins is known to mediate gene regulation in response in cytokines, growth factors, stress signals, bacterial and viral infections [22]. Moerover, the AP-1 transcription factor has been linked to a many cellular processes including cell transformation, proliferation, differentiation and apoptosis [23]. From these reports, we expected that LYPD6B might also be involved in these important cell processes including cell proliferation in ovarian cancer cells.

We examined the relative effect of LYPD6B knockdown in

OVCAR 3 ovarian cancer cells. We used four different lentiviral shRNA particles for LYPD6B knockdown and these shRNAs targeted different sequences within LYPD6B; shRNA #1 targeted exon 6 to 7 and shRNA #3, #4 and #5 targeted exon 9. Although these targeted different sequence all are predicted to knockdown all three variants of LYPD6B and are not in regions specific to any splice form. qPCR results demonstrated that LYPD6B transcripts were 90-40 % knocked down in shRNA transfected OVCAR 3 cells as compared to the parental cell (Figure 7A). This result was confirmed by immunoblotting of LYPD6B protein levels (Figure 7B).

We did not observe any morphological changes in in vitro following lentiviral mediated shRNA knock-down of LYPD6B in OVCAR3 cells (data not shown). Because the previous study implicated LYPD6B in AP1 mediated in growth and stress responses we examined the proliferation of OVCAR 3 with and without knockdown. We noted no significant changes in proliferation rates for LYPD6B knockdown cells even in those cells with greater than 90% knock-down as measured by qRT-PCR (Figure 7C). Similarly we noted no differences for LYPD6B knockdown cells compared to parental cells and non-target shRNA transducted cells using several other cell based assays including 2D-colony formation assay, scratch migration assay and boyden chamber cell invasion assay. (data not shown).

| Affymetrix Id | GENE | Description | R[1] | Signal[2] |
|---|---|---|---|---|
| 228360_at | LYPD6B | LY6/PLAUR domain containing 6B | 1.00 | 1773 |
| 208891_at | DUSP6 | dual specificity phosphatase 6 | 0.58 | 2435 |
| 208892_s_at | DUSP6 | dual specificity phosphatase 6 | 0.57 | 1752 |
| 204973_at | GJB1 | gap junction protein, beta 1, 32kDa | 0.55 | 398 |
| 39549_at | NPAS2 | neuronal PAS domain protein 2 | 0.53 | 570 |
| 225098_at | ABI2 | abl-interactor 2 | 0.53 | 1405 |
| 231928_at | HES2 | hairy and enhancer of split 2 (Drosophila) | 0.52 | 100 |
| 217848_s_at | PPA1 | pyrophosphatase (inorganic) 1 | 0.52 | 16066 |
| 225506_at | KIAA1468 | KIAA1468 | 0.49 | 392 |
| 208893_s_at | DUSP6 | dual specificity phosphatase 6 | 0.49 | 188 |
| 229002_at | FAM69B | family with sequence similarity 69, member B | 0.49 | 477 |
| 1554020_at | BICD1 | bicaudal D homolog 1 (Drosophila) | 0.49 | 1123 |
| 203935_at | ACVR1 | activin A receptor, type I | 0.49 | 1765 |
| 201411_s_at | PLEKHB2 | pleckstrin homology domain containing, family B member 2 | 0.49 | 898 |
| 210688_s_at | CPT1A | carnitine palmitoyltransferase 1A (liver) | 0.49 | 183 |
| 227764_at | LYPD6 | LY6/PLAUR domain containing 6 | 0.49 | 122 |
| 222499_at | MRPS16 | mitochondrial ribosomal protein S16 | 0.48 | 681 |
| 209272_at | NAB1 | NGFI-A binding protein 1 (EGR1 binding protein 1) | 0.48 | 4702 |
| 224516_s_at | CXXC5 | CXXC finger 5 | 0.48 | 6962 |
| 233955_x_at | CXXC5 | CXXC finger 5 | 0.48 | 6485 |
| 211596_s_at | LRIG1 | leucine-rich repeats and immunoglobulin-like domains 1 | 0.48 | 4128 |
| 201410_at | PLEKHB2 | pleckstrin homology domain containing, family B member 2 | 0.47 | 2957 |
| 226424_at | CAPS | calcyphosine | 0.47 | 826 |
| 225717_at | KIAA1715 | KIAA1715 | 0.47 | 350 |
| 208634_s_at | MACF1 | microtubule-actin crosslinking factor 1 | 0.47 | 4005 |
| 203414_at | MMD | monocyte to macrophage differentiation-associated | 0.47 | 1248 |
| 218274_s_at | ANKZF1 | ankyrin repeat and zinc finger domain containing 1 | 0.46 | 477 |
| 213462_at | NPAS2 | neuronal PAS domain protein 2 | 0.45 | 473 |
| 212641_at | HIVEP2 | HIV type I enhancer binding protein 2 | 0.45 | 310 |
| 1561536_at | LOC283435 | hypothetical protein LOC283435 | -0.45 | 119 |
| 227785_at | SDCCAG8 | serologically defined colon cancer antigen 8 | -0.45 | 430 |
| 212255_s_at | ATP2C1 | ATPase, Ca++ transporting, type 2C, member 1 | -0.46 | 2304 |
| 1570552_at | C18orf50 | chromosome 18 open reading frame 50 | -0.46 | 115 |
| 211340_s_at | MCAM | melanoma cell adhesion molecule | -0.46 | 1250 |
| 1566967_at | SPRY4 | sprouty homolog 4 isoform 1 | -0.46 | 118 |
| 222303_at | ETS2 | v-ets erythroblastosis virus E26 oncogene | -0.46 | 177 |
| 203934_at | KDR | kinase insert domain receptor | -0.47 | 373 |
| 209210_s_at | FERMT2 | fermitin family homolog 2 (Drosophila) | -0.48 | 2549 |
| 204948_s_at | FST | follistatin | -0.48 | 639 |
| 215513_at | HYMAI | hydatidiform mole associated and imprinted | -0.48 | 107 |
| 218595_s_at | HEATR1 | HEAT repeat containing 1 | -0.48 | 3414 |
| 64899_at | LPPR2 | lipid phosphate phosphatase-related protein type 2 | -0.51 | 263 |
| 210184_at | ITGAX | integrin, alpha X | -0.51 | 139 |
| 226847_at | FST | follistatin | -0.52 | 520 |
| 236875_at | LOC 100130705 | hypothetical protein LOC100130705 | -0.55 | 104 |

[1] Correlation coefficient to LYPD6B; [2] Geometric average signal of cancers

**Table 4:** Transcripts correlated with LYPD6B in 53 stage IIIC serous ovarian cancers in data set (2) [20].

| Affymetrix Id | GENE | Description | R (1)[1] | R (2)[2] |
|---|---|---|---|---|
| 228360_at | LYPD6B | LY6/PLAUR domain containing 6B | 1.00 | 1.00 |
| 209272_at | NAB1 | NGFI-A binding protein 1 (EGR1 binding protein 1) | 0.73 | 0.48 |
| 204973_at | GJB1 | gap junction protein, beta 1, 32kDa | 0.71 | 0.55 |
| 211596_s_at | LRIG1 | leucine-rich repeats and immunoglobulin-like domains 1 | 0.67 | 0.48 |
| 233955_x_at | CXXC5 | CXXC finger 5 | 0.56 | 0.48 |
| 217848_s_at | PP | pyrophosphatase (inorganic) | 0.56 | 0.52 |
| 224516_s_at | CXXC5 | CXXC finger 5 | 0.55 | 0.48 |
| 208893_s_at | DUSP6 | dual specificity phosphatase 6 | 0.54 | 0.49 |
| 208891_at | DUSP6 | dual specificity phosphatase 6 | 0.51 | 0.58 |
| 227764_at | MGC52057 | hypothetical protein MGC52057 | 0.49 | 0.49 |
| 213462_at | NPAS2 | neuronal PAS domain protein 2 | 0.48 | 0.45 |
| 208892_s_at | DUSP6 | dual specificity phosphatase 6 | 0.48 | 0.57 |
| 39549_at | NPAS2 | neuronal PAS domain protein 2 | 0.47 | 0.53 |
| 208634_s_at | MACF1 | microtubule-actin crosslinking factor 1 | 0.44 | 0.47 |
| 211340_s_at | MCAM | melanoma cell adhesion molecule | -0.49 | -0.46 |
| 203934_at | KDR | kinase insert domain receptor | -0.63 | -0.47 |
| 209210_s_at | PLEKHC1 | pleckstrin homology domain containing, family C member 1 | -0.63 | -0.48 |

[1] Correlation coefficient to LYPD6B in data set (1); [2] Correlation coefficient to LYPD6B in data set (2)

**Table 5:** Transcripts correlated with LYPD6B in stage IIIC/IV serous ovarian cancers common to data sets (1) and (2).

| Affymetrix Id | GENE | Description | R[1] | Signal[2] |
|---|---|---|---|---|
| 228360_at | LYPD6B | LY6/PLAUR domain containing 6B | 1.00 | 941 |
| 209436_at | SPON1 | spondin 1, extracellular matrix protein | 0.56 | 10451 |
| 213994_s_at | SPON1 | spondin 1, extracellular matrix protein | 0.54 | 5044 |
| 209437_s_at | SPON1 | spondin 1, extracellular matrix protein | 0.53 | 2248 |
| 213993_at | SPON1 | spondin 1, extracellular matrix protein | 0.53 | 2206 |
| 227763_at | LYPD6 | LY6/PLAUR domain containing 6 | 0.53 | 97 |
| 1554863_s_at | DOK5 | docking protein 5 | 0.52 | 469 |
| 214844_s_at | DOK5 | docking protein 5 | 0.50 | 1799 |
| 227764_at | LYPD6 | LY6/PLAUR domain containing 6 | 0.49 | 293 |
| 224488_s_at | SPON1 | spondin 1, extracellular matrix protein | 0.48 | 231 |
| 206414_s_at | ASAP2 | ArfGAP with SH3 domain, ankyrin repeat and PH domain 2 | 0.47 | 2454 |
| 212192_at | KCTD12 | potassium channel tetramerisation domain containing 12 | 0.46 | 6783 |
| 212909_at | LYPD1 | LY6/PLAUR domain containing 1 | 0.45 | 2337 |
| 35666_at | SEMA3F | sema domain, immunoglobulin domain, short basic domain, secreted, 3F | 0.44 | 1611 |
| 218847_at | IGF2BP2 | insulin-like growth factor 2 mRNA binding protein 2 | 0.44 | 1708 |
| 209272_at | NAB1 | NGFI-A binding protein 1 (EGR1 binding protein 1) | 0.43 | 3240 |
| 205227_at | IL1RAP | interleukin 1 receptor accessory protein | 0.43 | 821 |
| 204733_at | KLK6 | kallikrein-related peptidase 6 | 0.43 | 1854 |
| 219230_at | TMEM100 | transmembrane protein 100 | 0.43 | 601 |
| 205593_s_at | PDE9A | phosphodiesterase 9A | 0.42 | 500 |
| 202668_at | EFNB2 | ephrin-B2 | 0.42 | 2312 |
| 244523_at | MMD | monocyte to macrophage differentiation-associated | 0.42 | 182 |
| 205473_at | ATP6V1B1 | ATPase, $H^+$ transporting, lysosomal 56/58kDa, V1 subunit B1 | 0.42 | 728 |
| 224516_s_at | CXXC5 | CXXC finger 5 | 0.41 | 5420 |
| 208025_s_at | HMGA2 | high mobility group AT-hook 2 | 0.41 | 910 |
| 232476_at | ASAP2 | ArfGAP with SH3 domain, ankyrin repeat and PH | 0.41 | 497 |
| 219836_at | ZBED2 | zinc finger, BED-type containing 2 | 0.41 | 776 |
| 239028_at | LYPD6 | LY6/PLAUR domain containing 6 | 0.40 | 119 |
| 211596_s_at | LRIG1 | leucine-rich repeats and immunoglobulin-like domains 1 | 0.40 | 3909 |
| 221655_x_at | EPS8L1 | EPS8-like 1 | 0.40 | 887 |
| 213386_at | C9orf125 | chromosome 9 open reading frame 125 | 0.40 | 272 |
| 226021_at | RDH10 | retinol dehydrogenase 10 (all-trans) | -0.42 | 695 |
| 232579_at | LOC 100134229 | hypothetical protein LOC100134229 | -0.44 | 260 |

[1]Correlation coefficient to LYPD6B; [2] Geometric average signal of cancers

**Table 6:** Transcripts correlated with LYPD6B in data set (3) [21]. Genes correlated to LYPD6B at correlation coefficient > 0.4 or <-.4 in 189 cases.

## LYPD6B correlated transcripts

Because we saw no LYPD6B mediated effects in several cell based assays we attempted to futher understand any cellular relations to LYPD6B by performing some comparative bioinformatics. We queried genome wide array data for the transcripts correlated with LYPD6B expression profile in an attempt to identify co-regulated genes relevant

to ovarian cancer biology. These correlations were examined in three data sets: (1) Our own stage IIIC/IV serous cancer derived data described above, (2) Affymetrix array data available at gene expression omnibus website deposited by Mok et al. (GSE18521) [20] and (3) Affymetrix array data deposited by Tothill et al. (GSE9899) [21]. The data set (2) includes 53 advanced stage serous ovarian cancers; and the data set (3) includes both serous and endometrioid histologic types including 145 advanced stage and 26 early stage malignant cases and 18 borderline cases. Thus the number of cancer cases used for correlation analysis are 20, 53 and 189 for data sets (1), (2) and (3) respectively. Initially the correlations of LYPD6B expression profile with each of the transcripts on array were evaluated. The genes were then sorted by the correlation coefficient (R) and highly co-regulated genes were identified from top and bottom of the list by setting threshold levels of R. The highest value of R obtained for the three data sets varied largely. The data set (1) indicated 23 transcripts correlated at $R > 0.75$ and 8 transcripts anti-correlated at $R < -0.75$ (Table 3). Among these LEM domain containing 1 (LEMD1) is most similar to LYPD6B ($R = 0.87$) and carbohydrate sulfotransferase 12 (CHST12) is the most anti-correlated ($R = -0.9$). Interestingly, none of the LYPD family members were found in this list because of smaller values of R. The best correlated family member with LYPD6B is LYPD1 at $R = 0.7$. Similar analysis on data set (2) of Mok et al. indicated highest $R = 0.58$. There were 45 transcripts at $R > 0.45$ or $R < -0.45$ (Table 4) in which dual specificity phosphatase 6 (DUSP6) is at the top ($R = 0.58$). A search for common transcripts to data sets (1) and (2) at $R > 0.45$ or $R < -0.45$ in both data sets identified 12 genes including DUSP6, CXXC finger 5 (CXXC5), gap junction protein, beta 1, 32kDa (GJB1) and leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) (Table 5). The expression profiles of 189 cases in data set (3) indicate 33 transcripts either positively or negatively correlate with LYPD6B expression pattern at $R > 0.4$ or $< -0.4$ (Table 6). Spondin 1 (SPON1) and LYPD6 have the best correlations with LYPD6B in this data set. CXXC5 and LRIG1 are found commonly in all three data sets though their correlation coefficients are not high. CXXC5 is a BMP regulated modulator of WNT signaling. LRIG1 is integral membrane protein and a negative regulator of growth factor signaling. These findings point out that LYPD6B expression is co-regulated with genes involved in diverse pathways of ovarian cancers.

In conclusion, we indentified that the LYPD family member genes LYPD6B and LYPD1 were over-expressed in advanced stage serous ovarian cancer. We found that LYPD6B was localized on the cell membrane of OVCAR 3 cells as was expected from protein domain prediction analysis. The function of LYPD6B remains unclear in ovarian cells. Initial knockdown studies of LYPD6B in OVCAR 3 cells did not change several in vitro phenotypes including cell morphology, proliferation, migration or viability. Further study is required to reveal the function of LYPD6B in general and in ovarian cancer specifically. The cell surface nature of the molecule may enhance the attractiveness of LYPD6B use in future biomarker studies related to ovarian cancer detection or recurrence.

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