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14. ABSTRACT
The overarching goal of my laboratory is to investigate whether circulating LO, a novel class of atypically large (1-10μm diameter), bioactive extracellular vesicles (EVs), which are released by highly invasive and metastatic amoeboid tumor cells in the plasma, and contain abundant RNA, miRNA, DNA, and protein cargo, report clinically relevant information and tumor-specific genomic alterations, thus representing a valuable alternative and/or complement to other technologies proposed as a means of liquid biopsy. Collectively our findings indicate that DNA analyses of LO in blood (plasma) may provide a faithful representation of the genome of the tumor cells of origin. Because we have performed also comparative analysis with other EVs and shown that *LO are a source of high quality and abundant DNA and they contain the whole genome of donor tumor cells*, our overall objective is to **test whether enumeration and genomic profiling of LO circulating in patient blood allow early detection of metastatic PC and identification of clinically significant PC-specific genomic aberrations**, thereby overcoming the current limitations of the "liquid biopsy"

15. SUBJECT TERMS
Metastatic Castration Resistant Prostate Cancer, Extracellular Vesicles, Extracellular DNA, Large Oncosomes, Next Generation Sequencing, Liquid Biopsy, digital droplet PCR

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Introduction

We were the first to identify a novel class of atypically large (1-10μm diameter), bioactive extracellular vesicles (EVs), referred to as large oncosomes (LO) [1], which are released by amoeboid tumor cells, which are highly invasive and metastatic, as a consequence of DIAPH3 silencing [1-4]. These EVs contain signaling proteins and upon paracrine transfer to other cells can induce functional changes that are relevant to malignant tumor cell behavior [1, 2]. We have developed a suite of methods and assembled several lines of evidence that LO can be identified in tumor tissues and in plasma [1, 4, 5]. LO contain abundant RNA, miRNA, DNA, and protein cargo, and can be purified from biological fluids (i.e. plasma and urine). The molecular makeup of the LO tends to reflect the cell of origin. The overarching goal of my laboratory is to investigate whether circulating LO report clinically relevant information and tumor-specific genomic alterations, thus representing a valuable alternative and/or complement to other technologies proposed as a means of liquid biopsy. We are therefore characterizing the molecular content of LO. With funding by the NIH-NCI to profile the palmitoyl proteome of circulating LO by mass spectrometry, and by the DoD to characterize the DNA in LO by next generation sequencing, we are mostly focused on prostate cancer (PC). Our effort is highly significant because it might result in establishing a strategy for 1) early detection of high-risk PC, 2) early detection of metastatic CRPC, 3) identification of actionable tumor-derived genomic alterations in patient plasma for guided treatment. Our study is supported by preliminary results demonstrating that LO contain double stranded (ds) DNA that spans all chromosomes and recapitulates the genomic alterations of the donor cell. Furthermore, the mean somatic copy number aberrations (SCNA) of LO-derived DNA mirrors the mean SCNA of the genomic DNA of the cells of origin (PC3). Collectively our findings at the time of our proposal submission suggest that DNA analyses of LO in blood (plasma) may provide a faithful representation of the genome of the tumor cells of origin. Because we have performed also comparative analysis with other EVs and shown that *LO are a source of high quality and abundant DNA and they contain the whole genome of donor tumor cells*, our overall objective is to **test whether enumeration and genomic profiling of LO circulating in patient blood allow early detection of metastatic PC and identification of clinically significant PC-specific genomic aberrations,** thereby overcoming the current limitations of the "liquid biopsy" and changing the landscape of precision medicine.

Keywords
Prostate Cancer, Metastatic Castration Resistant Prostate Cancer, Extracellular Vesicles, Extracellular DNA, Large Oncosomes, Next Generation Sequencing, Liquid Biopsy, digital droplet PCR

Accomplishments

In this first year of the DoD funded project, we have performed extensive *in vitro* and *in vivo* experiments that will be enclosed in a manuscript that is in preparation. First of all, we aimed to confirm our initial findings that LO in plasma can provide an alternative source of tumor-derived DNA. We anticipated challenges to detect SCNA in this setting, because, unlike detecting SCNA of human cell DNA in mouse plasma, human plasma can contain human DNA originating from normal cells that might reduce the signal-to-noise-ratio. We isolated LO from plasma of PC patients with metastatic disease and tested SCNA. The assay used for LO in mouse plasma and in PC3 cells (**Figure 1a and b**) cannot distinguish tumor DNA from normal DNA, which will dilute the SCNA signals from tumor DNA (so-called "needle in a haystack problem"). Also, the amount of DNA can be very limited, so an efficient assay that requires only a small amount of DNA is needed.

We isolated large EV fractions containing LO from plasma specimens of 12 prostate cancer patients. As controls, we isolated the same biochemical fractions from the plasma of healthy individuals. We then extracted DNA from these fractions using DNeasy Blood & Tissue Kit (50)

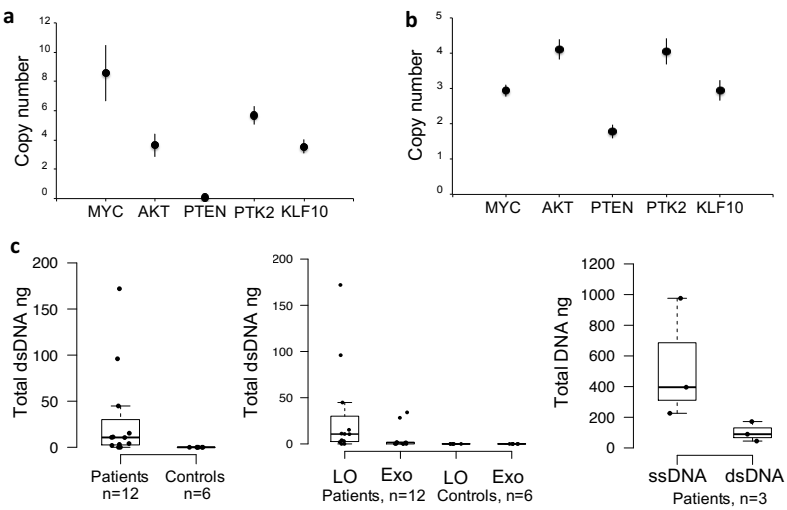

Figure 1. a) SCNA of MYC, AKT, PTEN, PTK2, and KLF10 in PC3-derived LO were measured by dPCR. Each target gene as normalized to gene reference RNaseP. **b)** SCNA alterations of MYC, AKT, PTEN, PTK2, and KLF10 were validated by dPCR in mouse plasma-derived LO, following intracardiac injection of PC3 cells. Each target gene as normalized to gene reference RNaseP. **c)** LO-derived DNA was detected in prostate cancer patient plasma but not in the plasma of healthy individuals. EVs were isolated from 1 ml of plasma.

(Qiagen, #69504). The amounts of DNA extracted from the large EV fractions were significantly higher in patient preparations than preparations from healthy individuals (Figure 1c). This result is consistent with our previous studies that have shown increased numbers of LO in patients with aggressive disease than in patients with low-risk prostate cancer and in healthy individuals [5].

Using digital PCR (dPCR), we measured the *MYC/PTEN* copy number ratio. These two genes are known to undergo genomic loss and amplification, respectively, which occurs at high frequency in patients with metastatic PC. Such an assay, if successfully validated in large number of samples, could potentially detect the instability of tumor genomes (copy number imbalance) in a wide range of prostate tumors. In the *in vitro* and mouse experiments (**Figure 1a and b**), we measured the copy number of a target gene (such as *MYC* or *PTEN*) and a reference gene (RNaseP) in a single dPCR chip, which allows us to determine the copy number ratio (*MYC/PTEN*) indirectly. Instead, for human samples, we developed an assay to measure *MYC/PTEN* ratio directly in one chip, thus eliminating the noise introduced by the indirect comparison (of *MYC* or *PTEN* versus RNaseP) and leveraging precious DNA samples from patients. Our results are not quantitative, in that they do not contain precise information about the exact copy number of either gene. Also, it is not possible to determine whether the copy number imbalance is due to *MYC* amplification or *PTEN* deletion. However, our results suggest that there is an imbalance about the *MYC/PTEN* ratio, which can be due to either MYC amplification or PTEN deletion. We found this ratio to be higher in cancer versus benign samples and the difference is significant (**Figure 2-3**.

More in details, we tested the *MYC/PTEN* ratio using variable amount of DNA from ENCODE normal cells (IMR90 primary fibroblast, ATCC® Number: CCL-186™, and GM12878 lymphoblastoid cell line, ATCC® Number: NIST-8398™). In the eight independent experiments with the template DNA between 2.5 ng and 20 ng, the *MYC/PTEN* ratio was almost equal to 1 (**Figure 2a**). We further evaluated the consistency of detecting copy number imbalance between different amounts of template DNA using DNA from PC3 10K fraction. In PC3, *PTEN* is deleted in homozygosity from intron 2 to the telomeric side but retained DNA from exon 2 to the centromeric side (not shown). Using the Taqman probe designed for the intron 1, we can evaluate the *MYC/PTEN* ratio in PC3. *MYC/PTEN* ratio was consistent between duplicates with 1 ng (3.2 and 3.4) and 5 ng template DNA (2.9 and 2.9) (**Figure 2b**). However, the ratio deviated between duplicates with 0.2 ng DNA (2.2 and 2.5), indicating that at least 1 ng DNA is needed to consistently call the ratio.

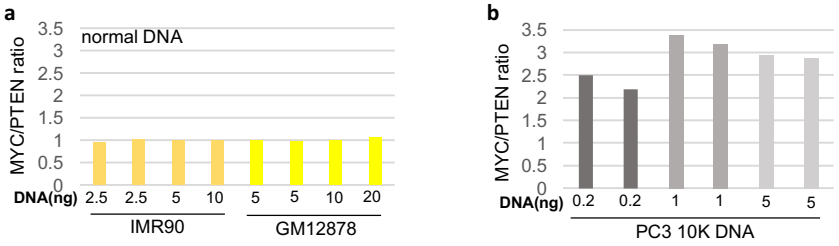

Figure 2. a) MYC/PTEN ratio in normal cell-derived DNA was validated by dPCR using different amounts of template DNA. **b)** MYC/PTEN copy number imbalance in PC3-deived LO. was assessed by dPCR using different amounts of template LO DNA.

Using this methodology, we evaluated *MYC/PTEN* ratio in a subset of patient samples **(Figure 3)**. We performed the experiments in triplicate. We also had a case in which DNA from two independent blood draws (two independent visits to clinic) was available to us through our collaboration with Dr. Posadas. We found that *MYC/PTEN* ratio was frequently above 1 in patients' DNA. In one case (Patient 2), we saw a small but significant deviation in all three independent chips. In another case, deviations are larger and consistent between two independent blood draws (Patient 25a and 25b). In this case, *MYC/PTEN* ratio fluctuated between experiments (Patient 25a: 1.39-1.61-1.24, and 25b: 1.91-2.23-2.06). The limited amount of template DNA for these experiments may explain the fluctuation.

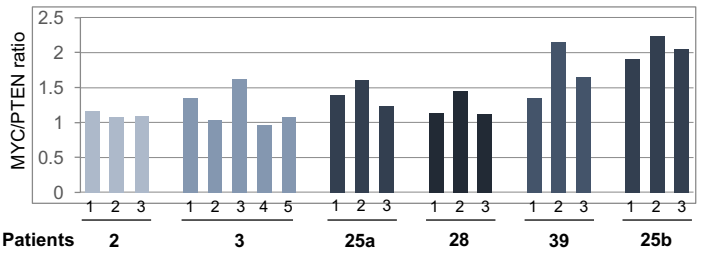

Figure 3. MYC/PTEN copy number imbalance was assessed by dPCR in LO isolated from 1 ml of metastatic prostate cancer patient plasma. Numbers 1-3 for patient samples 2, 25a and 25b refer to the number of replicates.

These results may indicate that LO provides an ideal material for cancer diagnosis. Since genome instability is a hallmark of tumors, the principle of the assay could be applicable to plasma LO in patients with many cancer types.

We also performed the very first large-scale DNA analysis of EVs isolated from the plasma of one patient with metastatic PCa. A Comprehensive Genomic Profiling (CGP) was performed, in collaboration with Foundation Medicine, to

interrogate patient plasma-derived EVs about Prostate Cancer Specific SCNA. The commercial name of the test is FoundationOne, and this is a validated test to detect all classes of genomic alterations in more than 300 cancer-related genes, including select introns from more than 25 genes often rearranged or altered in solid tumors.

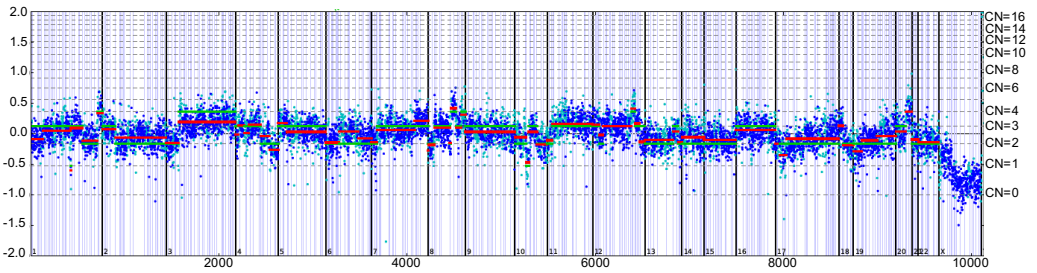

Figure 4. Prostate cancer-specific SCNA detected by genomic profiling of EVs isolated from 1 ml of metastatic prostate cancer patient plasma.

What we learned from this single patient data is that we can perform this large scale analysis with 97% of exons covered at >100x, and at Median Unique Exon Coverage of 191x. We found 1) evidence for ***TMPRSS2-ERG* fusion** at lower limit of detection, 2) evidence for aneuploidy characteristic specific to prostate cancer (8p loss, 8q amplified), 3) Two 'likely' cancer-specific short indels were detected: *BRCA2* (44% MAF) and *PBRM1* (7% MAF), 4) evidence for *PTEN* loss. Importantly, a comparison with the CGP performed on the matched tumor tissue of the same patient displayed similar genomic alterations.

In parallel experiments, we have identified molecules, whose alterations are responsible for nuclear membrane instability as a mechanism for LO formation and/or for the export of DNA in LO:

Evidence that NM instability results in nuclear blebbing and the shedding of EVs that contain nuclear material. In preliminary studies, we have found that DIAPH3 silencing results in the formation of nuclear blebs, and in the simultaneous extracellular release of LO, which are a subclass of particularly large EV originating from amoeboid cancer cells [2]. During this first year of the DoD funding, we demonstrated that DIAPH3 silencing is followed by dysregulation and altered localization of the inner nuclear envelope protein emerin, and by the emergence of nuclear blebs that seem to give rise to cytosolic vesicles (Fig. 5). Analysis of EV isolated from the culture medium of these cell lines indicates they contain emerin as well as genomic DNA (Fig. 5). Based on these findings, we hypothesize that NM instability and the resulting formation of nuclear blebs could be a potential mechanism by which the genomic DNA enters in EV. We also hypothesize that we can mark the nuclear origin of these particles with emerin and possibly other nuclear proteins (add in tissues also). These findings suggest that NM instability may be detectable using a blood test in a liquid biopsy format. A mechanism where EV carrying genomic DNA arise from nuclear blebs has not been described.

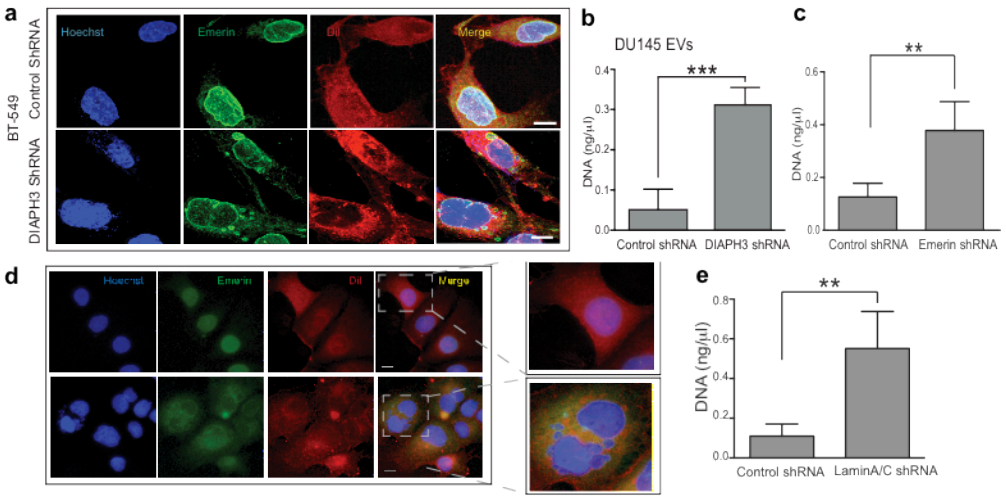

Figure 5. DIAPH3 silencing induce emerin mislocalization and nuclear membrane instability. **a)** Confocal 3D images of DU145 prostate cancer cells transfected with control shRNA or DIAPH3 shRNA. DNA (Hoeschst), emerin (FITC) and membrane (1,1'-Dioctadecyl-3,3,3'3'-Tetramethyilindocarbocyanine Perclorate, DiI) staining are shown, scale bar 10mm. **b,c)** DIAPH3 silencing induces the shedding of EVs containing DNA. The DNA concentration in EVs was determined by Qubit. **d)** Confocal 3D images of DU145 prostate cancer cells transfected with control shRNA or nuclear envelope regulator LaminA/C shRNA inducing similar effects to the ones induced by DIAPH3 silencing. Also Lamin A/C silencing induces increased shedding of EVs containing DNA.

Importantly, in collaboration with Andries Zijlstra, at Vanderbilt University, we have demonstrated that ***this same feature is present in high grade PC in humans and is significantly higher in cancer versus normal and correlates with biochemical recurrence (Fig 6)***. From these data, we *hypothesize* that emerin ***can serve as a biomarker for the highly migratory and metastatic amoeboid phenotype***, and that it can be used to detect and measure nuclear membrane

instability in cancer tissues and plasma. They also suggest that this is the mechanism that allow the DNA to enter EVs. This is a hypothesis that we will investigate under different funding.

Impact

LO-derived DNA reports the genomic makeup of the tumor and support LO as the fraction of plasma DNA to be interrogated for tumor-derived genomic alterations. This finding has profound implications on the field because it provides a valid alternative to other strategies that are being implemented for liquid biopsy. The impact of our study is very high also because the concepts originating from our findings can be applied to human tissues for diagnosis, prognosis and prediction of treatment response. More specifically, because low levels of DIAPH3 increase sensitivity to taxanes [2, 6] and because patients with low DIAPH3 expression in their tumor tissue show better responses to taxane-containing chemotherapy than patients with high DIAPH3 expression [2, 6], these findings suggest that measurement of emerin mislocalization, as a surrogate for NM instability, can serve as a biomarker for taxane sensitivity. This structural feature has never been described in PC.

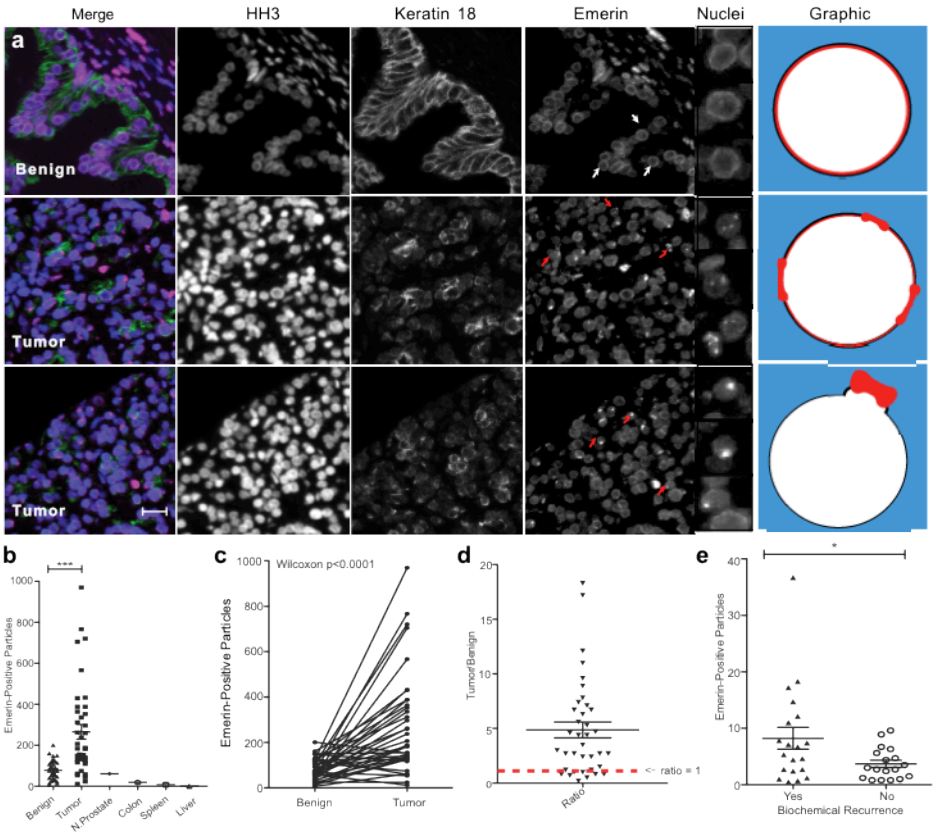

Figure 6. Emerin mislocalization and emerin-positive particles are associated with biochemical recurrence. a) IF imaging of a human prostate cancer TMA stained with the indicated antibodies demonstrates the presence of emerin positive structures outside the cancer cell nuclei, suggesting that features of nuclear membrane instability can be identified in vivo. **b-e)** the emerin features correlate to prostate cancer and biochemical recurrence

Changes/Problems: There are not substantial changes to the specific aims and tasks. However, we have delayed the execution of the large-scale profiling of LO from the proposed numbers of patient plasma samples. This is due in part to the time that was spent to obtain IRB approval, and in part to the time that was spent in the optimization and standardization of DNA analysis in LO. We plan to perform these experiments in year 2.

Products:

Nine manuscripts were published under this first year of funding, one more is under consideration in Nature Medicine, and two more are in preparation.

1. Gardiner C, **Di Vizio D**, Sahoo S, Théry C, Witwer KW, Wauben M, Hill AF. Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. J Extracell Vesicles. 2016 31; 5:32945

2. Meehan B, Rak J, Di Vizio D. (2016) Oncosomes, large and small: what they are, where they came from? *J Extracell Vesicles*. Sep 27;5:33109

3. Conley A, Minciacchi VR, Lee DH, Knudsen BS, Karlan BY, Citrigno L, Viglietto G, Tewari M, Freeman MR, Demichelis F, **Di Vizio D**. High-throughput sequencing of two populations of extracellular vesicles provides an mRNA signature that can be detected in the circulation of breast cancer patients. RNA Biol. 2017 14(3):305-316

4. Torri A, Carpi D, Bulgheroni E, Crosti MC, Moro M, Gruarin P, Rossi RL, Rossetti G, **Di Vizio D**, Hoxha M, Bollati V, Gagliani C, Tacchetti C, Paroni M, Geginat J, Corti L, Venegoni L, Berti E, Pagani M, Matarese G, Abrignani S, de Candia P. Extracellular microRNA signature of human helper T cell subsets in health and autoimmunity. J Biol Chem. 2017 292(7):2903-2915

5. Pathan M, Keerthikumar S, Chisanga D, Alessandro R, Askenase P, Camussi G, Clayton A, Collino F, **Di Vizio D**, Falcon JM, Fontana S, Gho YS, Hendrix A, Nolte-t Hoen E, Iraci N, Kislinger T, Kowal J, Kurochkin IV, Leonardi T, Liang Y, Llorente A, Lotvall J, Lunavat TR, Lyden D, Monteleone F, Overbye A, Panaretakis T, Patel T, Pedro A, Pluchino S, Principe S, Ronquist G, Royo F, Sahoo S, Peinado Selgas H, Spinelli C, Stensballe A, Thery C, van Herwijnen M, Wauben M, Welton J and Mathivanan S. A novel community needs software for functional enrichment analysis. J Extracell Vesicles. 2017 6(1):1321455
6. Minciacchi V.R., Spinelli C., Reis-Sobreiro M., Huang W., Cavallini L., You S., Zandian M., Adam R.M., Posadas E.M., Viglietto G., Freeman M.R., Cocucci E., Bhowmick N.A., **Di Vizio D**. MYC mediates large oncosome-induced fibroblast reprogramming in prostate cancer. Cancer Res. 2017 77(9):2306-2317
7. Schillaci O, Fontana S, Monteleone F, Taverna S, Bi Bella MA, **Di Vizio D**, Alessandro R. Exosomes from metastatic cancer cells transfer amoeboid phenotype to non-metastatic cells and increase endothelial permeability: their emerging role in tumor heterogeneity. Scientific Reports 2017 7(1):4711. doi: 10.1038/s41598-017-05002-y.
8. Minciacchi VR, Zijlstra A, Rubin MA, **Di Vizio D.** Extracellular vesicles for liquid biopsy in prostate cancer: where are we and where are we headed? Prostate Cancer and Prostatic Disease 2017, 20(3):251-258
9. Lau AN, Israelsen WJ, Roper J, Sinnamon MJ, Georgeon L, Dayton TL, Hillis AL, Yilmaz OH, **Di Vizio D**, Hung KE, Vander Heiden MG. (2017) PKM2 is not required for colon cancer initiated by APC loss, *Cancer & Metabolism, in press*

Participants and other collaborating organizations
Tatyana Vagner: post-doctoral fellow
Edwin Posadas: collaborator
Stephen Freedland: collaborator
Andrea Sboner: collaborator

References

1. Di Vizio, D., et al., *Large oncosomes in human prostate cancer tissues and in the circulation of mice with metastatic disease.* Am J Pathol, 2012. **181**(5): p. 1573-84.
2. Di Vizio, D., et al., *Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease.* Cancer Res, 2009. **69**(13): p. 5601-9.
3. Hager, M.H., et al., *DIAPH3 governs the cellular transition to the amoeboid tumour phenotype.* EMBO Mol Med, 2012. **4**(8): p. 743-60.
4. Morello, M., et al., *Large oncosomes mediate intercellular transfer of functional microRNA.* Cell Cycle, 2013. **in press**.
5. Minciacchi, V.R., et al., *Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles.* Oncotarget, 2015. **6**(13): p. 11327-41.
6. Morley, S., et al., *Regulation of microtubule dynamics by DIAPH3 influences amoeboid tumor cell mechanics and sensitivity to taxanes.* Sci Rep, 2015. **5**: p. 12136.