Characterizing the role of LARP1 in cancer

By

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies (FGPS), University of Ottawa in partial fulfillment of the requirements for degree of Master of Science

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A B S T R A C T

Protein synthesis is frequently dysregulated in cancer cells; such conditions are known to favor aberrant cell growth and proliferation which lead to cancer. LARP1 is a novel target of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, a circuitry often hyperactivated in cancer which regulates cell growth and proliferation primarily through the regulation of protein synthesis. I aimed to determine if LARP1 plays a role in cancer progression by comparing its expression in normal versus cancer tissues. My results demonstrate that LARP1 expression is altered (lost or overexpressed) in various cancers and correlates with cancer patients survival. My systematic bioinformatics assessment, the results of my functional assays assessing the effect of LARP1 knockdown on cancer cells, together with my antibody validation do not only provide new insights for its role in cancer progression and mRNA translation, but also emphasizes the potential of LARP1 as a cancer therapeutic target.

A C K N O W L E D G E M E N T S

I would like to thank my supervisor, Dr. Tommy Alain for his guidance and remarkable trust in my success, his openness and his constant encouraging smile. Dr. Alain has demonstrated an incredible attention for my training and personal improvement, providing great opportunities, and unforgettable lifetime advice and research tips.

A sincere gratitude to the members of my thesis advisory committee, Dr. Martin Holcik and Dr. Seung Hwan Lee who have provided priceless guidelines, thoughtful suggestions for the design of experiments. I would also like to extend my sincere thanks to my second supervisor, Dr. David Stojdl for his support throughout my training in the lab at the CHEO and at the University of Ottawa.

Special thanks to Dr. David Grynspan from the Pathology department at the CHEO and to Dr. Eric Belanger from the Ottawa Hospital for providing tissues and guidance for immunoassays on human tissues, thank you for your critical analysis and insightful experimental suggestions.

Special thanks to Dr. Christina Addison, Dr. Barbara Vanderhyden, Dr. Jim Dimitroulakos and Dr. Luc Sabourin from the Ottawa Hospital Research institute for providing cell lines and critical insights.

Thanks to Dr. Daneshmand Manijeh from the Histology Core Facility of the University of Ottawa for reviewing my immunohistochemistry results.

I am very thankful for all the support I have received from the staff including principal investigators, research technicians and students at the Apoptosis Research Centre at the Children's Hospital of Eastern Ontario Research Institute.

I would also like to thank all the members of the Tommy Alain Laboratory. I am very grateful for your assistance and collaborative spirit. Thanks for sharing your time and expertise for my training and my improvement. Special thanks to Dr. Tyson Graber for reviewing my thesis.

I am thankful to my family and friends who continually support and pray with me. No words can express my gratitude to my best friend and wife, Linda. Thanks for your love and support. Thanks for always being there. To our little boy, Jean Justin Kabambi, born in the middle of my training. To my father, Dr Justin Vital Kabambi who left us few days before the beginning of my lab experiments.

Lastly and specifically, I thank GOD for being faithful even when we are not. Thanks for being my source and my shepherd, my fortress and my friend, my source of science, wisdom and excellence.

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LIST OF ABBREVIATIONS

4EBP	4E-Binding Protein
SKT	Serine/Threonine protein kinase
AML	Acute Myeloid Leukemia
APS	Ammonium persulfate
AR	Androgen Receptor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
c-myc	Cellular myelocytomatosis oncogene
CO2	Carbon dioxide
CRPC	Castration Resistant Prostate Cancer
ddH2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenedietholaminetetra-acetic acid
eIF	Eukaryotic Initiation Factor
EGR2	Early growth response 2
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein

HeLa	Henrietta Lacks cell line
HSV	Herpes simplex virus
IHC	Immunohistochemistry
IRES	Internal Ribosome-Entry Site
kDa	KiloDalton
LARP	LA-related protein
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
NC	Nitrocellulose
NEPC	Neuroendocrine Prostate Cancer
PABP	Poly(A)-Binding Protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PFA	Paraformaldehyde
PSA	Prostate-specific antigen
RB	Retinoblastoma
RPMI	1640 Roswell Park Memorial Institute 1640 medium
RRM	RNA Recognition Motif
S6K1	S6 Protein Kinase 1
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
shRNA	Short hairpin RNA
UTR	Untranslated Region
VSV	Vesicular stomatitis virus

1. INTRODUCTION

Cancer is a considerably devastating cause of death in the world. In Canada, the number of men dying of cancer is tragically increasing every year. For the year 2015, it was estimated more than 100,000 Canadian men will be diagnosed with cancer; among those more than 40,000 will die from it[1]. In Canada, the most common types of cancer are lung, breast, colorectal and prostate cancer. Nearly 25% of all new cancer cases in Canadian men are accounted by prostate cancer. The latter is the second most common cancer in men worldwide [1]. As for many heterogeneous diseases, early detection of prostate cancer is critical for better survival. Currently, the main methods used to detect prostate cancer are PSA (prostate specific antigen), blood test and digital rectal exam. These diagnostic strategies are not only unpleasant and invasive; but they have been reported to have poor accuracy. While diagnosis of prostate cancer at an early stage (stage I/II) leads to over 90% progression-free-survival, the likelihood of a successful cure is almost null when patients are diagnosed at stage III and stage IV [2, 3]. Considerable efforts are being made to identify new highly selective and sensitive biomarkers and develop drugs against selected genes and pathways involved in cancer progression. In that respect, genes and proteins that reveal alterations such as mutations, loss or gain of functions and variation of expression when normal cells are compared to cancer cells are of a tremendous importance when aiming to detect and treat early stage cancers.

As prostate cancer represents a burden for men, ovarian cancer is recognized not only as the fourth most common cause of cancer-related deaths in the world [4] but also as the deadliest of all gynecological cancers [5]. The remarkable case-fatality rate reputed for ovarian cancer can be partly if not mainly explained by late diagnosis. Epithelial ovarian cancer is the most

[1] Statistics Canada Catalogue no. 82-624-X

common type of ovarian cancer and is detected in postmenopausal women for the majority of the cases [6].

Diagnosis and treatment of prostate and ovarian cancer

Currently, the detection of PSA in serum remains the main early screening strategy for prostate cancer patients [7]. Although several studies have mentioned that most of early-detected prostate cancer cases are not lethal, it is true that PSA screening strategy has considerably reduced the presentation of prostate cancer cases at high-grade. Consequently, methods currently used to establish prostate cancer prognostic and risk of progression are considered as suboptimal. Moreover, there is a considerably large number of men who are overtreated due to non-optimal prostate cancer diagnosis, leading to catastrophic financial struggles for their families and for the health care system [8-11].

Treatment of prostate cancer is often done by stage/phase of the disease. Standard treatment for stage I to stage III consists of surveillance, prostatectomy and radiotherapy. This group does not include high-risk patients of stage III. Surgery for androgen ablation and chemical castration are used for patients in stage IV and high-risk patients in stage III. This approach seems to result in relatively sustained remission. However, genomic mutations in androgen receptor lead to castration resistance. As such, castration resistant prostate cancer (CRPC) is among the most targeted types of cancer regarding new drugs development. Considering the heterogeneity and the complexity of tumor cells, several strategies are being explored to develop anti-prostate cancer drugs: anti-androgens, protein kinase inhibitors, chemotherapy, growth factor antagonists and cancer vaccines. Unfortunately, only very few have reached phase III clinical trials (**Figure 1**).

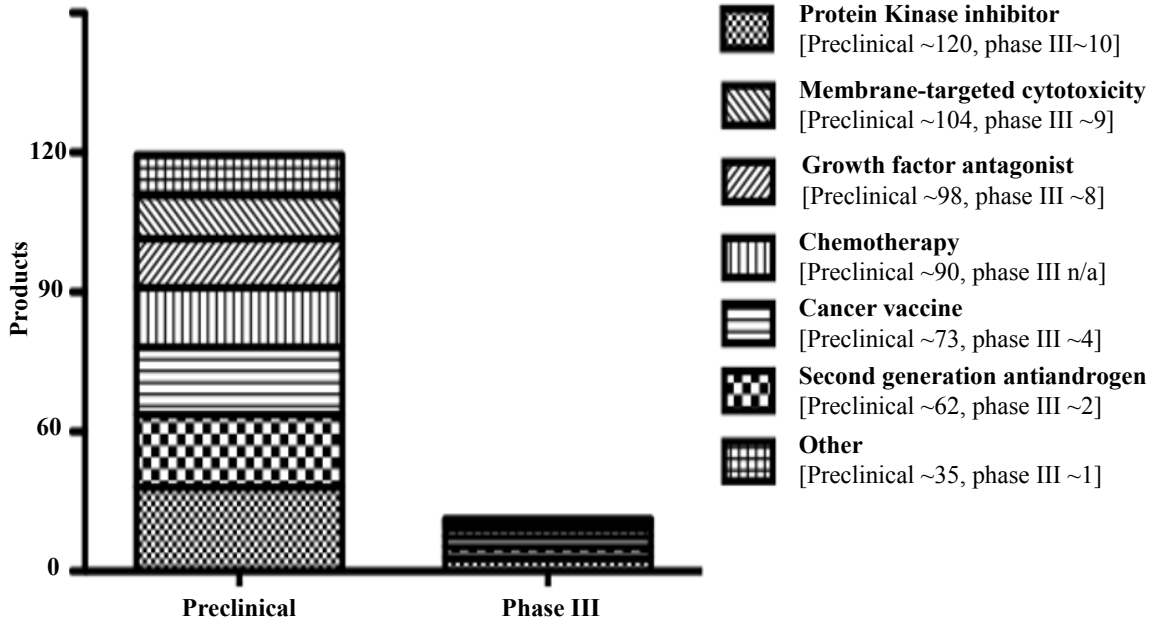

Figure 1. Pipeline of selected anti-prostate cancer products separated by stage of development and by the mechanism of action. 'Others' refers to apoptosis activators, cell membrane disruptors and histone deacetylase complex inhibitors. Modified from the GBI Research Proprietary Database (pipeline molecules) [12].

Although it's well recognized that early events at the molecular level in the development of epithelial ovarian cancer can be key for the success of any therapeutic and diagnosis strategies, very little is known about them and no considerably sensitive or specific screening tests for ovarian cancer have been developed. Unfortunately, more than 75% of ovarian cancer patients receive a diagnosis in advanced stage. Given that the chances of surviving depend closely on when the cancer is diagnosed, the detection and right interpretation of symptoms on the one hand and the development of reliable biomarkers on the other hand are the best strategies to improve ovarian cancer patient survival. Currently, the main screening methods used to detect ovarian cancer are the pelvic exam, the transvaginal sonography and the CA-125 blood test [13]. Combination of taxane / platinum based therapy and combined cytoreductive surgical interventions are the main strategies used to treat epithelial ovarian cancer [14].

The issue related to reliable biomarkers is not exclusive to prostate and ovarian cancer, used here to exemplify the problem, but englobes almost all cancers. In this context, it is noteworthy that a huge effort is being made to characterize main cancer progression mechanisms to allow a more specific targeting of cancer cells.

The Hallmarks of Cancer

Cancer can be defined as a complex disease characterized by uncontrollable cell growth and proliferation. Early cancer biologists have assumed that certain specific and similar molecular networks are responsible for cancer cell proliferation and cancer metastasis. Six common physiological phenomena have been identified and proposed by Douglas Hanahan and Robert Weinberg as the hallmarks of cancer [15]. The first six hallmarks identified were: self-sufficiency in growth signal, insensitivity to anti-growth signals, evading programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion/metastasis.

Some tumors have the ability to demonstrate all the six hallmarks, due to specific mutations in "key" genes (such as p53 which controls at least four of the six hallmarks) whereas most tumors require more than a single mutation to progress. In recent years, more hallmarks have been added to conceptualize the multistep of cancer progression with each step reflecting different genetic alterations: deregulated metabolism, evading the immune system, genome instability and inflammation. The current metaphor and strategies used to describe and target cancer cells depend mainly on the understanding of those hallmarks (**Figure 2**).

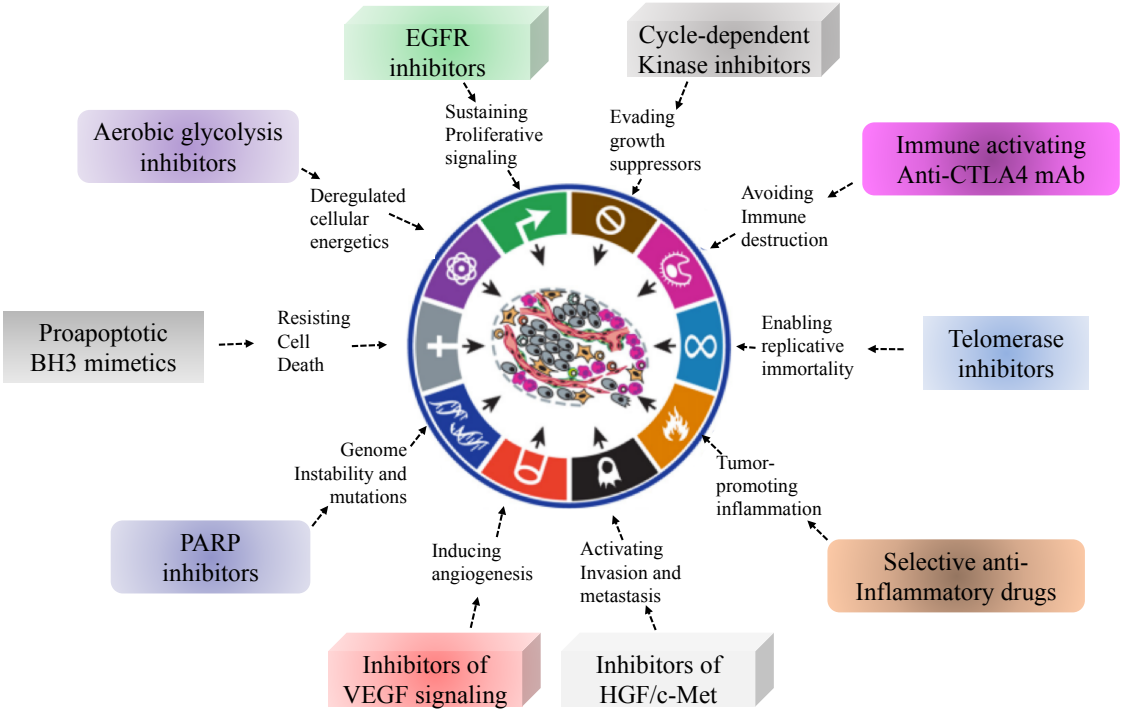

Figure 2. Illustrative Therapeutic Targeting of the Hallmarks of Cancer. Drugs that react to the acquired abilities required for tumor growth and cancer progression are being developed and tested for clinical trials with humans. Modified from Hanahan and Weinberg's hallmarks of cancer [15].

Role of growth factors in cancer progression

Generally speaking, the individual behavior of the cell depends not only on the cell itself, but includes and is related to external factors and signals from the microenvironment within the tissue or organ. External growth factors transfer the message via specific signals, which will repress (inhibit) or enhance (promote) the expression of a given number of genes (**Figure 3**). Diffusible growth factors, extracellular matrix proteins and cell-cell adhesion/interaction molecules are simple examples of those growth factors, which trigger cell division. Dependence on external growth factors is an homeostatic mechanism of paramount importance for the control of cell behaviour within a tissue.

Niepet et al. [16] have shown that soluble growth factors within the microenvironment play an important role in the development and invasion of cancer cells. Thus, growth factors have influence on metastasis and responsiveness of tumors to targeted therapies. Moreoever, tumor growth and tumor progression can be enhanced by potential cross-talks of different growth factor signaling pathways. By crossing each other and other protumorigenic factors, these pathways can amplify their functions to the extent of acquiring novel protumorigenic properties to facilitate tumor progression. The p53-mediated chromosomal instability is a good example to illustrate this process. Nevertheless, other oncogenic pathways do target the bioactivity of growth factor pathways (e.g. the expression of some growth factor components induced by p53).

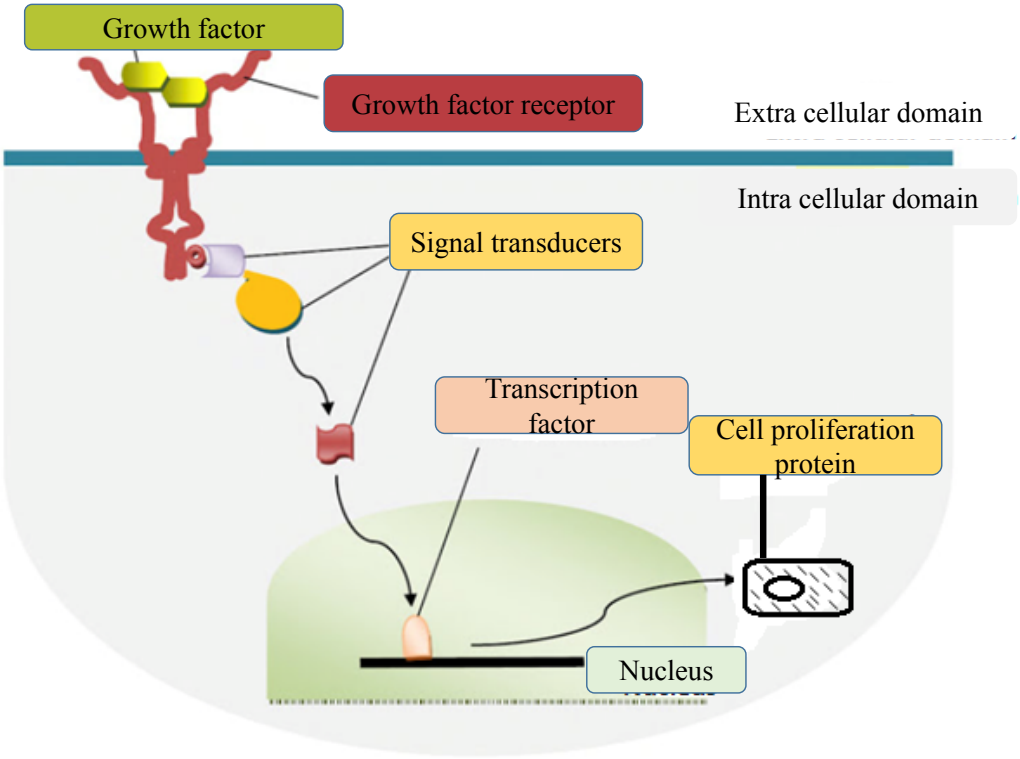

Figure 3. Simplified representation of the components of a typical growth factor signaling cascade. Hormones and cell-bound signals are example of growth factors that stimulate cell proliferation. Signal transducers are proteins (and other molecules) that transmit the received signal from the receptor to other intracellular components involved in cell proliferation. Illustration modified from "The Biology of Cancer"[15].

At the cellular level, disruptions in a handful of key pathways are common to many different types of cancer. There are three levels of changes commonly observed at the cellular level in cancer cells by which they achieve certain growth factor autonomy:

1. Changes in the level of extracellular growth signals;

2. Changes in the level of transcellular signals (cell surface receptors): by being mutated or changed in number, cell surface receptors transducing growth-stimulatory signals into the cell can be transformed into cancer cells. The tyrosine kinase family is the most common group of receptors involved in several types of cancer [15];

3. Changes in the level of intracellular pathways related to proliferation [16, 17].

Cancer pathways

A certain limited number of pathways regulate cell proliferation, cell differentiation and the survival of cells by transmitting and integrating signals from growth factors, hormones, cell-cell and cell-matrix interactions. These "pathways" turn into "cancer pathways" once they are deregulated by inappropriate activation or deregulated inactivation. Many proto-oncogenes and tumor suppressors operate upon or within those so-called "cancer pathways".

Tyrosine Kinases

Tyrosine kinases are a specific group of protein kinases involved in the signal transduction cascades wherein they help transmit extracellular signals through the cell membrane to the cytoplasm. These signals, when introduced to the nucleus, affect and modify gene expression [18, 19]. Protein kinase inhibitors are among the leading strategies used for the development of anti-prostate cancer drugs (**Figure 1**). Phosphorylation at tyrosine residues plays a role in

many different cellular functions at the protein level such as enzyme activity, subcellular localization, and interaction between molecules.

Another interesting group of protein kinases is the serine/threonine (ser/thr) kinase (STK) family. STKs phosphorylate the OH group of the amino acids serine and threonine and their expression level is altered in many cancers. The role of PI3-kinases (signal transducers that phosphorylate the 3 position OH group of the inositol ring of phosphatidylinositol) in key cellular functions such as cell growth, proliferation, motility and survival is attributed to their ability to activate specific protein kinases such as AKT in the PI3K/AKT/mTOR pathway.

The mTOR pathway

The mammalian target of rapamycin (mTOR) is a STK from the PI3K-related kinase (PIKK) subfamily. PIKKs share similarity with PI3Ks in many functions with the exception of their lipid kinase activity. The kinase mTOR was such named when it was discovered to be the target of the drug rapamycin. Rapamycin is a macrolide produced by Streptomyces hygroscopius with fungal properties discovered in 1975 [20]. The name rapamycin derives from Rapa Nui (Easter Island) where the compound was discovered. Shortly after its discovery, research confirmed the immunosuppressive properties of rapamycin. Intriguingly, rapamycin was suspected to have anticancer properties, but the mechanism by which rapamycin was able to stop cancer remained unknown for almost two decades [21-23].

Rapamycin inhibits the highly conserved protein kinase target of rapamycin (TOR) complex 1 but does not bind the mTOR complex 2. The development of ATP-competitive mTOR inhibitors, which target both mTORC1 and mTORC2, shed light about the ability of mTORC1 to phosphorylate eIF4E-binding protein (4E-BP1) and demonstrated that this mechanism is rapamycin insensitive [24, 25].

mTORC1 recruits substrates through regulatory-associated protein of mTOR (RAPTOR or RPTOR), which binds a TOR signalling (TOS) motif in substrate proteins (for example the ribosomal S6 kinase S6K and the eIF4E-binding proteins 4E-BPs). Thus, rapamycin can inhibit the activity of mTORC1 towards some substrates by interfering in a selective manner with the disposition of a bound substrate. As mentionned previsouly, mTORC1 (mTOR complex 1) signaling pathway is often hyperactivated in cancer (dysregulated in 80% of malignancies) and regulates cell growth and proliferation partly by controlling the translation of specific mRNA transcripts [25-27].

The role of RNA-binding proteins in cancer progression

Genomic evolution, with increasing influence of RNA biology, has led to the discovery of microRNAs (miRNAs), non-coding RNAs (ncRNAs) and RNA-binding proteins (RBPs) as dominant factors of gene expression [28-30]. RBPs have crucial roles in various cellular processes such as cellular function, transport and localization. They especially play a major role in post-transcriptional control of RNAs, such as splicing, polyadenylation, mRNA stabilization, mRNA localization and translation. Although RBPs have a demonstrated and crucial role in post-transcriptional regulation of gene expression, relatively few RBPs have been systematically studied [31, 32].

RNA-binding proteins (RBPs) regulate the decay kinetics and translational efficiency of mRNA transcripts by accelerating their degradation or prolonging their cytoplasmic half-life. Thus, the abundance of mRNAs and their encoded proteins may be altered in a manner that is independent of gene transcription. It’s worth mentioning that RBPs themselves are regulated by growth factors and cell signals. Taken together, these two post transcriptional mechanisms related to RBP (control of mRNA transcripts and regulation by growth factors) enable the cell to rapidly adjust levels of protein expression in response to intrinsic and extracellular signals.

The La-related proteins family

LA-related proteins (LARP family) are RNA-binding proteins sharing a conserved sequence of 90 amino acids called the LA motif (LAM) signature similar to that of Genuine La protein. This motif was named after the so called Genuine La protein. Seven LA-related proteins (LARPs) have been identified in humans: LARP1 (LARP1a called LARP1 and LARP1b also called LARP2), LARP3 (also called SS-B or Genuine La), LARP4 (also called LARP4a), LARP4b (also called LARP5), LARP6 and LARP7. Bousquet-Antonelli and Deragon [33] have previously described the structure of the LARPs distinguishing their common structures from their divergent features. An illustration describing the structure of the LARP family based on literature review [34, 35] is presented in **Figure 4.**

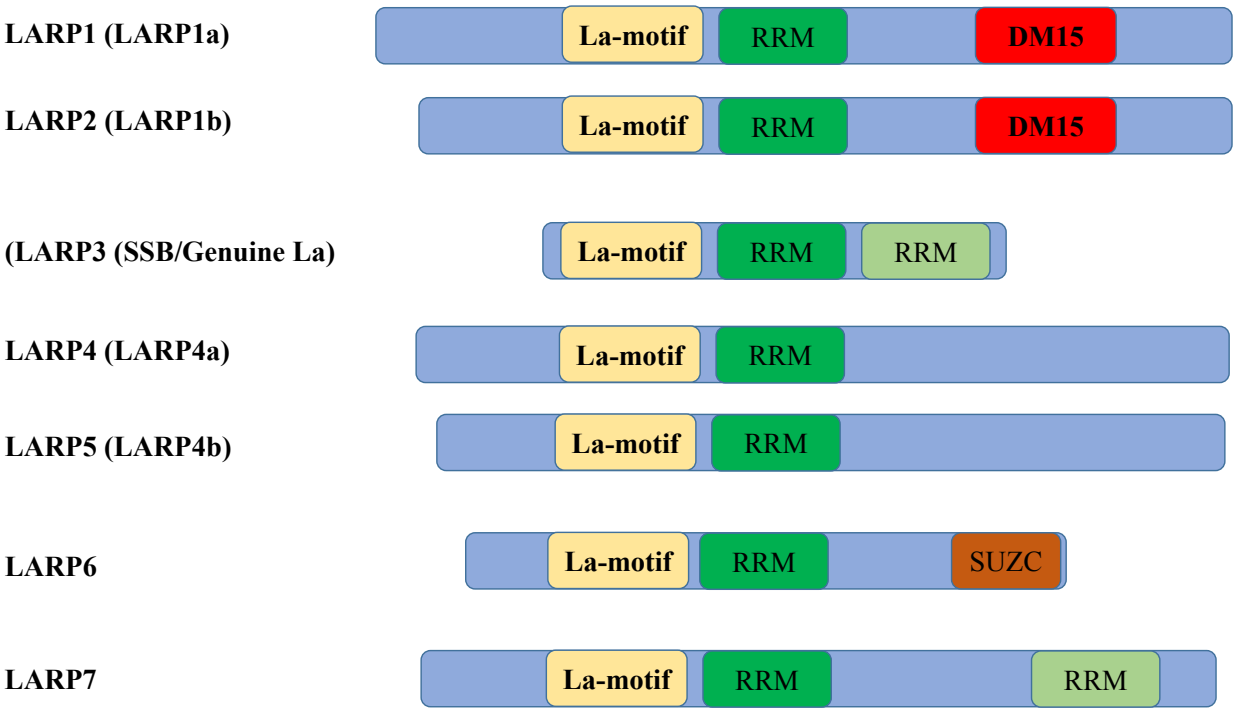

Figure 4. Domain architecture of the LA-related proteins. All LARPs share the La motif (La-motif) which is located near the N-terminus for LARP3 but more centrally placed for the other LARPs. LARP3 and LARP7 carry two RNA-recognition motifs (RRM) while other LARPs bear 1 RRM located closed to the LAM and some RNA recognition-like motifs (RRM-L). LARP1a and LARP1b share the DM15-repeat containing region (DM15 also called LARP1 motif). LARP6 carries a specific domain at the C-terminus named the SUZ-C domain (SUZ-C). Modified from Stavraka and Blagden [35].

Unlike other RNA-binding proteins, the members of the LA-related protein family do not carry an enzymatic domain. However, all the LARPs bear RNA recognition motif which is usually associated with the La-motif and named together as the "La module". Some LARPs such as LARP3 and LARP7 carry more than one RRM while others carry an atypical RNA recognition -like motif (RRM-L). Interestingly, LARP1a and LARP1b share a specific motif at the C-terminal which is comprised of amino acid repeats known as the DM15 region (also called the LARP1 motif as it is exclusively found in LARP1a and LARP1b) [33]. Surprisingly, a 36 amino acids sequence found at the C-terminal of other RNA-binding proteins and named SUZ-C domain is found in LARP6. Although the role of the SUZ-C domain has not been clearly defined for LARP6, it has been previously proposed that this domain is utilized by RBPs for subcellular localization. LARP4 an LARP5 share a non-typical N-terminal domain called the PAM2w domain (not included in the illustration). This domain has been previously proposed to bind the polyadenylate binding protein (PABP) [36].

Role of La-related proteins in cancer

In this study, the expression level of LARP1 in normal tissues versus their adjacent cancer tissues was analyzed using different databases. A comparison of cancer patients' survival based on the expression of LARP1 was also established (See **Bioinformatics results**). Other LARPs have also been studied in the context of cancer survival. Many studies have suggested that LARP7 is a potential tumor suppressor in gastric cancer while LARP3 has been shown to play an oncogenic role in hepatocellular cancer cells. The role and proposed mechanisms of interaction for all LARPs are presented in **Table 1**. Some functions and mechanisms were omitted as they are beyond the scope of this study. Although there are two paralogs of LARP1 (LARP1a and LARP1b), most studies referring to LARP1 only consider LARP1a. I have not found any published work describing the role of LARP1b in cancer.

Table 1. Summarized putative roles of LARPs and mechanism of action.

Isoform	Proposed mechanisms	Proposed role /function	References
LARP1 (1096 aa)	*Target of the mTORC1 *Binds TOP mRNAs *binds PABP	*Repressor of translation *Activator of TOP mRNA translation *mRNA stability	Fonseca et al. 2015 [26] Aoki and Burrows et al., 2013[37] Tcherkezian et al. 2014 [54]
LARP2 (914aa)	*Role in de novo synthesis of milk fatty acid	*no link with cancer established	Duchemin et al. 2017 [99]
LARP3 (408aa)	*Target IRES-mediated genes such as Cyclin D1, BiP and MDM2.	*Mostly oncogenic *squamous cancers of the head and neck and cervix. *Hepatocellular cancer cell *Myeloproliferative diseases	Kuehnert et al.2015 [38] Kim et al., 2001 [39]
LARP4 (724aa)	*Interacts with the scaffold protein Ribosome-Associated Receptor for Activated C Kinase 1 (RACK1) *Interacts with cytosolic PABP	*Knockdown promotes cancer cell migration in prostate cancer cell line (PC3)	Bai et al.,2011 [40]
LARP5 (738aa)	*Interacts with PABP *Suggested role in suppressing tumor suppressor cell cycle factors p16 and p19	*Inhibition attenuated self-renewal *Knockdown caused cell cycle arrest.	Angenstein et al. 2002 [41] Schäffler et al.2010 [42] Zhang et al.2015 [43]
LARP6 (491aa)	*binds two regions of collagen mRNAs *shuttles between the nucleus and cytoplasm	*enhances cell proliferation, cell invasion *enhances angiogenesis and tumor growth *potential proto-oncogene	Shao et al.,2012 [44]
LARP7 (582aa)	*binds RNA polymerase III transcripts (7SK RNA) *indirectly suppresses mRNA transcription (Through 7SK RNA) *indirectly influences alternative splicing (Through P-TEFb)	*potential tumour suppressor in gastric cancer *Inhibition enhanced tumour progression and metastasis in MCF10A cells	Cheng et al. 2012 [45] He et al.2008 [46] Ji et al. 2014 [47] Mori et al.2002 [48]

However, tremendous amount of information describing the link between LARPs and patient cancer survival, related genetic alterations or protein expression can be found in publically available databases such as the Cancer Genome Atlas, the Human Protein Atlas (www.proteinatlas.com), Oncomine (www.oncomine.org), Kmplot (www.kmplot.com) and others.

mTOR/ LARP1 and TOP mRNA translation

The mammalian target of rapamycin complex 1 (mTORC1) is known to control mRNA translation through phosphorylation of its substrates. The eIF4E binding proteins (4E-BP1/2) and the ribosomal protein S6 kinase-1 (S6K1) are arguably the best characterized mTORC1 targets and represent negative and positive regulators of mRNA translation initiation, respectively [49]. Through successive phosphorylation by mTORC1, 4E-BPs reduce its affinity for the eukaryotic initiation factor 4E (eIF4E), freeing the latter to bind to the 7-methyl guanosine (m7Gppp) cap structure at the 5' end of cellular mRNAs. eIF4E promotes initiation of mRNA translation at the 5' cap by interacting with a scaffold protein eIF4G and the RNA helicase eIF4A, which together form the eIF4F complex. This step is crucial for the initiation of translation. Although S6K1 (when phosphorylated) plays also an important role in translation, it does not however interact directly with the formation of the eIF4F complex but rather interferes with the phosphorylation of key translational factor eEF2 [50-52].

The translational machinery is a crucial step of cell growth especially for tumor cell as controlling translation would result in controlling cell growth and proliferation, which is crucial for cancer progression. Foremost among the most important consequence of translation control by mTOR is the ability to regulate the synthesis of ribosomal proteins and other translation factors.

These ribosomal proteins and some other key translation factors are encoded by a specific group of mRNA transcripts named Terminal Olygopyrimidine (TOP) mRNAs. These mRNAs are so called because they all carry a 5' TOP motif downstream of their m7Gppp mRNA cap. Ribosomal proteins (and a number of translation factors) are encoded by a subgroup of mRNAs containing a 5'TOP motif immediately downstream of the m7Gppp cap structure. Various research groups have validated approximately 100 TOP mRNAs, with the majority encoding proteins involved in the translational apparatus. These include five translation elongation factors, nearly all the 80 ribosomal proteins, three translation initiation factors, the poly (A)-binding protein (PABP), and other proteins that are not classified as translation factors [53].

All TOP mRNA transcripts share distinguishing characteristics among which the most important are:

1) Immediately downstream of the TOP motif there is always a CG-rich region;

2) An interrupted series of four to fifteen pyrimidines which come after a non-variable C residue at the mRNA cap site;

3) C and U residues are proportionally distributed within the pyrimidine region of the majority of the TOP mRNAs.

The biogenesis of ribosomal proteins encoded by TOP mRNAs is not only highly resource and energy-consuming but also depends on conditions such as hypoxia and nutritional uptake. This whole mechanism is considerably critical for the growth of tumor cells. The presence of the 5' TOP motif within these mRNAs has previously been shown to confer translation repression in conditions of nutrient or oxygen deprivation [54]. The mTOR complex 1 plays a seminal role in the regulation of TOP mRNA translation. Because the translational efficiency

of TOP mRNAs is almost maximal when active, or null when repressed; the control of TOP mRNA translation by mTORC1 is a potential regulatory mechanism of cell growth and cell proliferation [54, 55]. The translation of TOP mRNA transcripts has been shown to be activated by different growth stimuli such as insulin. Tang and colleagues have demonstrated that serum and amino acids can at least partially signal to TOP mRNA translation [56]. Thus, the elevation of TOP mRNA translation efficiency induced by different stimuli at least partly satisfies the requirement of increased ability to induce cell growth and protein synthesis. [57-59].

Several studies have demonstrated that LARP1 deficiency selectively affects the recruitment of TOP mRNAs to polysomes [26, 54]. Even though a decrease abundance of proteins encoded by TOP mRNAs has been reported in LARP1 silenced cells, Aoki and colleagues argued that this can be explained simply by the reduced number of TOP mRNA transcripts in LARP1-deficient cells [54, 60].

There is a discrepancy on the exact role played by LARP1 in the translation of TOP mRNAs. Some authors have described LARP1 as an activator of TOP mRNAs [54] while others argue that LARP1 selectively represses the translation of TOP mRNAs [26, 98].

As for now, all proposed mechanisms of LARP1 involvement in the stability and abundance of TOP mRNA transcripts would require further validation and clarification based on in-vitro and in-vivo experiments. However, the main authors of the field agree that LARP1 is a relevant putative candidate as the missing link between mTOR complex 1 and the activity of 5'TOP motif of TOP mRNA transcripts. **Table 2** summarizes a selective literature review on the putative mechanism of TOP mRNA translation regulated by mTOR through LARP1.

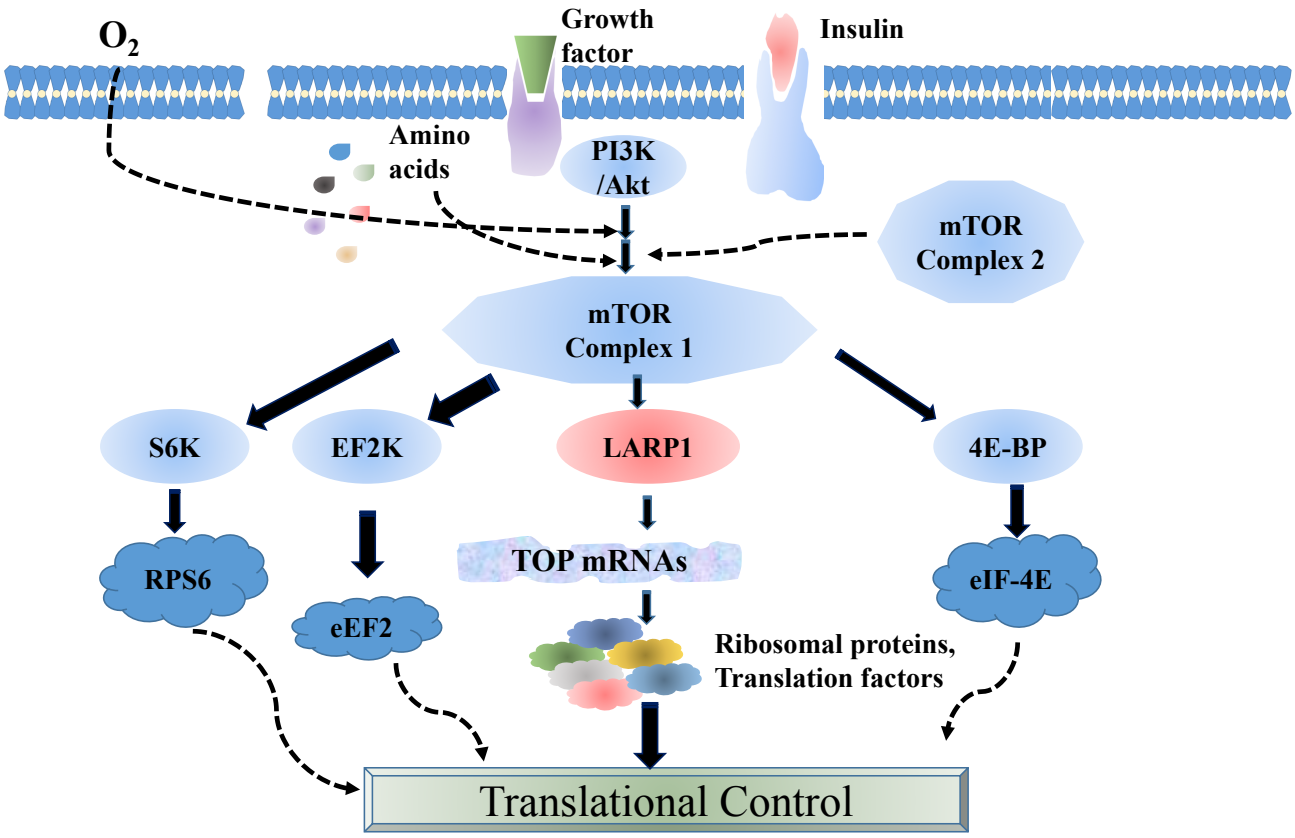

Figure 5. **LARP1 could be the missing link between mTOR pathway and the translation of TOP mRNA.** Pathways transducing external signals to the translational apparatus, through mTORC1 and TOP mRNAs are presented. For further details refer to the text. Modified from Mehuyas and Kahan, 2015 [60].

Table 2. Summary of research findings on LARP1 mechanism of action.

Publication	Observations	Conclusions
Natsume et al.2013 [37]	*LARP1 directly interact with poly(A) of TOP mRNAs *LARP1 binds the 5'cap of TOP mRNAs	LARP1 stabilizes the circular conformation of TOP mRNAs to sustain protein synthesis.
Roux et al.2014 [54]	*LARP1 binds PABP through the DM15 region *LARP1 interaction with cap (eIF4E) is a consequence of PABP interaction, not an independent process	*LARP1 has a positive effect on protein synthesis (activator). *LARP1 interacts with RAPTOR
Fonseca et al.2015 [26]	*LARP1 binds PABP *LARP1 interacts with RAPTOR	*LARP1 is a negative regulator (=Repressor) of TOP mRNAs translation *mTOR inhibition or nutrient starvation causes LARP1 to displace eIF4G from the eIF4F complex
Hong et al.2017 [61]	*non-phosphorylated LARP1 interacts with both 5' and 3'UTRs	*Non-phosphorylated LARP1 inhibits TOP mRNA translation *Phosphorylated LARP1 activates TOP mRNA translation *LARP1= phosphorylation-sensitive molecular switch used by mTOR to turn TOP mRNA translation off or on
Lahr et al.2017 [98]	*LARP1 directly binds the cap *LARP1 binds adjacent TOP motif of TOP mRNAs	*LARP1 is a specialized TOP mRNA cap-binding protein

Antibody Validation

Antibodies have been among the major tools used to study proteins in basic research. However, due to many issues caused by antibody staining failure, the scientific community has recently expressed an urgent need to extensively and thoroughly validate antibodies for each desired application. Uhlen and other distinguished co-authors have recently proposed a series of guidelines for antibody validation published in Nature Methods [62]. In their work, the group of Uhlen and colleagues suggested five main concepts for the validation of antibodies which include genetic validation strategies, independent antibody validation, orthogonal validation strategies, expression of tagged proteins and "other" strategies such as Immunocapture followed by mass spectrometry. It's strongly recommended that at least one of these main strategies be used to validate antibody for specific application. The discovery of the LARP1 as novel target of mTORC with important implication in the translation of TOP mRNAs and cell proliferation has received widespread attention from multiple companies. Thus, companies have generated LARP1 antibodies mostly for single application (mainly Western blotting or immunohistochemistry). Unfortunately, these antibodies have not yet been validated for multiple application.

Rationale and Hypothesis

Given that LARP1 is a novel target of mTOR downstream the PI3K/Akt/mTOR pathway and LARP1 is proposed to control the translation of TOP mRNAs; I hypothesize that LARP1 plays a role in cancer progression and its expression level correlates with patient survival. I organized the study as follows:

Objective 1: Characterize the expression level of LARP1 at the mRNA level and establish correlation with cancer patient survival.

Objective 2: Characterize LARP1 expression at the protein level comparing normal tissues versus adjacent cancer tissues.

Objective 3: Validate selected commercially available LARP1 antibodies for immunoassays.

Objective 4: Perform functional assays to determine the effect of LARP1 silencing on cancer cell lines.

The results should shed light on the potential role of LARP1 as a therapeutic target and eventually its function as a potential tumor suppressor or proto-oncogene.

2. MATERIALS AND METHODS

Materials, reagents and Equipments

Materials and equipments

All materials and equipment used for this project are listed in the appendices and presented in the methods.

Reagents

All reagents used in this project are listed in the appendices and presented in the methods.

Methods

Bioinformatics analysis

The Human Protein Atlas

In the preliminary work of this study, the aim was to elucidate the role of LARP1 in the progression of different cancers by characterizing it expression level for each cancer type. The cancer tissue atlas of the Human Protein Atlas (www.proteinatlas.org) [63] was used to make a comparison between the protein expression level of LARP1 and other proteins in different cancer tissues and their corresponding tissues. Selected pictures of human tissues stained with three different LARP1 primary antibodies: CAB01522 (Rabbit polyclonal LARP1, Atlas antibodies), HPA051319 (Polyclonal antibody against human LARP1, Sigma Aldrich) and HPA 054819 (Polyclonal antibody against human LARP1, Sigma Aldrich) are presented.

The Human Protein Atlas is a public database curating histological images of twenty of the most common types of cancer and 44 normal human tissues. In total, 216

immunohistochemical images of cancer samples were used to characterize the expression profile of different proteins.

Individual images were annotated by independent pathologists with an internal annotation bioinformatics tool, scoring the percentage positivity, the staining intensity and the staining localization. In this work, the Human Protein Atlas website was accessed from December 2015 to April 2016 using version 13.

Kaplan-Meier plotter (kmplot)

To determine the correlation between LARP1 expression level and cancer patients' survival, Kaplan-Meier plots from online available dataset were used where patients are separated by the expression level of the gene of interest. The Kaplan Meier Plotter (www.kmplot.com) is a publicly available integrative bioinformatic tool for data analysis that curates a plethora of gene expression data from Affymetrix microarrays from the Gene Expression Omnibus, the European Genome-phenome and The Cancer Genome Atlas [64]. KM Plotter is able to provide survival curves for more than 4,000 breast, almost 2,5000 lung, nearly 1,500 ovarian and more than 1,000 gastric cancer patients. Clinical data and related gene expression information are more than once updated every year using a PostgreSQL server.

In order to determine the robustness of survival analysis, all results presented in the curves are classified based on the sample number as: highly reliable (more than 500 samples), neutral (500-200 samples), preliminary (200-50) and explorative (less than 50 samples). Survival plots of cancer patients based on the expression level of the gene of interest can be sorted as overall survival (OS), progression free survival (PFS), Post-progression survival (PPS), Distant metastasis free survival (DMFS), Relapse free survival (RFS) and first progression (FP). As recommended on the website (www.kmplot.com), overall survival and progression

survival were used for ovarian cancer, and relapse free survival considered for breast cancer. Overall survival was used for lung and gastric cancer in this study.

For all survival plots produced, the hazard ratio with 95% confidence intervals and logrank P value are provided. KM Plotter was accessed from September 2015 to September 2016 for all analyses.

The Cancer Cell Line Encyclopedia (CCLE)

The GENE-E analysis tool from the link http://www.broadinstitute.org/ccle/home was interrogated to characterize the expression of LARP1 and some other selected genes in various cancer cell lines based on the mean Robust Multi-Array Average (RMA) expression. The comprehensive cancer cell line encyclopedia (CCLE) is a publicly available project aiming to conduct a detailed characterization of a considerably large panel of cancer cell lines. The CCLE offers access to the visualization of mRNA expression of a given gene in more than thousand cancer cell lines.

The Cancer Genome Atlas (TCGA) Cbioportal

The Cancer Genome Atlas (TCGA) database was interrogated to gather information regarding LARP1 genetic alterations occurring in various cancers. The TCGA Cbioportal is an open access database available publicly at http://www.cbioportal.org [65]. Moreover, valuable overall survival and disease-free survival differences can be compared between cancer patients separated by genetic alterations of LARP1 and other selected genes were produced from the TCGA Cbioportal if available. “Mutation and CNA (DNA copy-number alterations) and some other options were selected in the web interface of the TCGA cbioportal when available as query parameters. Fortunately, the Cbioportal data also offers information on the frequency and the location of mutations in analyzed protein domains. In general, gray bars

represent protein whole lengths with the size (number of amino acids) displayed at the bottom. Lines and dots represent the locations and frequencies of genes. Nonsense or frameshift mutations are represented by red and in-frame deletions are shown in green. Green, blue and red boxes represent the protein domains. No informed consent, neither statement of approval are required for these data, as they are obtained from an open access database (www.cbioportal.org).

Network analysis

In order to determine a predictable binding pattern of LARP1, the STRING database was interrogated to list the genes/proteins which are known or are predicted to interact with LARP1. The frequency of gene alteration is highlighted by color coded edges. STRING is a database which reassembles predicted and well-known protein-protein interactions. The interactions include both physical (direct) and functional (indirect) associations resulting knowledge transfer between organisms, from computer-based prediction and from interactions aggregated obtained from other (primary) databases (www.string-db.org)

ONCOMINE DATABASE

To further characterize the expression and function of LARP1 in cancers, Oncomine cancer Microarray database (http://www.oncomine.com) [66] was used to compare different tumor types with their normal tissue counterparts. To avoid confusion and wrong conclusions, only gene expression data from the same study comparing tumor and adjacent normal tissues were considered. This strategy offers the insurance that the same methodology was used for both studies. Results are presented in log form, median centered per array and the value of the standard deviation normalized as one per array. To be deemed overexpressed, the mean value of the gene in cancer tissues must be significantly higher than its mean value in the normal tissue counterpart.

GraphPad prism software (GraphPad Software, San Diego, USA) was used to analyze the results. The fold of induction higher or equal to 1.5 and t-test (p-value inferior or equal to 0.05) was used as statistical analysis for the analysis of gene expression determined using Oncomine.

Table3. Summary of the main databases used in this study

Database	Oncogenic data	Link
Kaplan-Meier plot	Survival analyses	www.kmplot.com
Cancer cell line Encyclopedia	mRNA expression of genes in cancer cell lines	www.broadinstitute.org/ccle/home
TCGA/Cbioportal	Gene alteration/Mutation, copy-number alterations, mRNA expression, protein/phosphoprotein level, survival analyses	www.cbioportal.org
Oncomine	mRNA expression (normal versus cancer tissues)	www.oncomine.org
The Human Protein Atlas	Protein expression Cancer versus normal cells	www.proteinatlas.org

Cell culture and LARP1 knockdown

Cell culture. The human embryonic kidney HEK293T and HeLa (human cervical adenocarcinoma) cell lines were obtained from American Tissue Culture Collection (Manassas, VA, USA). The OVCAR8 human ovarian cancer cell line was a gift from the Vanderhyden laboratory at the Ottawa Hospital Research institute (Ottawa, Ontario, Canada). Cells were maintained in medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin at 37º C under 5% CO2 conditions.

Transfection of Lentiviruses. Two different shRNAs sequences targeting the gene sequence of LARP1 (GenBank: NM_007764 were used as the interference sequences: shLARP1-

2(TRCN0000150984) and shLARP1-4 (TRCN0000152891). A non-related targeting sequence was also used as scrambled shSCR (SHC002). The corresponding shRNAs for LARP1 and shSCR were then inserted into the pLKO.1-puro vector system (7086 bp) and transfected together with pLP1 (8889 bp), pLP2 (4180 bp), and pLP/VSVG (5821 bp). 2.10^6 HEK293T cells were seeded into a 10cm dish and cultured in DMEM supplemented with 10% FBS 1% P/S. When the cells reached 75% confluency, 8 µg of each vector diluted in OPTIMEM medium was added to the cells using the PEI transfection system. After 5h, OPTIMEM was replaced by full growing medium (DMEM 10%FBS, 1%P/S). Filtered medium (0,45um) containing lentivirus was collected 48h and 72h later and the supernatant used for transduction or stored at -80 degree if not used directly.

Transduction. 0,3 $x10^6$ cells of the target cell line were seeded into 6 well plate and cultured at 37ºC, 5% CO2 until cells reached 75% confluency. After 2 washes with PBS 1X, a mix of 1mL of lentiviral supernatant and 1mL of growing medium were added to the cells. Polybrene (5µg/mL) was added to cells to enhance transduction efficiency. After 24h, transduction was repeated using the same procedure. Cells were transferred from 6well plate to 10cm dishes. Stably transfected cells were screened by puromycin selection (5 µg/mL) for 5days. LARP1 silencing was confirmed by Western blot and immunofluorescence.

SDS-PAGE and Western blotting

Cells were washed twice with cold PBS 1X and lysed with 1 mL or 500 µL RIPA buffer containing 50 mM NaF, 15mM Na_3VO_4, 0,5 mM DTT and 0,2 mL protease inhibitor. The supernatants were collected following centrifugation at 14,800 rpm for 30 min at 4°C. Protein concentration was determined using the Lowry protein assay (Spectra Max Microplate spectrophotometer, Molecular devices, serial number LN 02465). Equal amounts of proteins diluted in sample loading buffer (1:4) were loaded into 10% SDS-PAGE gels for 1 hour at

100V. Protein bands were transferred onto nitrocellulose (NC) membrane for 1 h at 100V and blocked by 5% skim milk in TBST for 1 h at room temperature. The membrane was then incubated with primary antibody on shaker at 4^{o} C overnight. Primary antibodies were diluted in 5% BSA 0, 01% NaN_3 in TBST and incubated for 1 h at room temperature or overnight at 4^{o} C. After three washes with TBTS for 5min each, membranes were incubated with peroxidase-conjugated goat Anti Rabbit/Anti-Mouse IgG (1:4000 in 5% milk in TBST) for 1 h at room temperature. The membranes were washed 3x5 min with TBST buffer and incubated 5 min in ECL visualization solution. Bound proteins were detected using films developed with KODA Film processor. Beta-actin or GAPDH were used as loading control.

Immunofluorescence staining

$3x10^5$ Cells were seeded in 6 well plate and cultured on glass coverslips for 16h. After wash with PBS, cells were fixed with 4% PFA in PHEM buffer (4% PFA, 25 mM HEPES, 60 mM PIPES, 4 mM MgCl, 10 mM EGTA). Autofluorescence was blocked with 50mM NH4Cl. Cells were blocked 30min with 5% BSA in PBS, then permeabilized 10 min with+ 0.1% Triton X-100 at room temperature. Samples were incubated first with LARP1 Antibody (rabbit, 1/100 in 1% BSA/PBS) overnight at 4°C then with alpha acetylated tubulin Ab (mouse,1/200 in 1% BSA/PBS) for 1h at RT. Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG were used as secondary antibody for LARP1 antibody and alpha acetylated tubulin antibody respectively. Cells were mounted with Acrytol mounting Medium (Electron Microscopy Sciences, catalog 1358, lot 170224). Immunofluorescence staining was analyzed using Olympus Fluoview 1000 confocal microscope and EVOS FL auto AMAFD1000 microscope. Four to six cell images were analyzed comparing LARP1 expression in scrambled (shSCR) and knockdown (shLARP1) cells; the relative staining (IF signal) was quantified using Image J software. Results are presented in the graph as the mean ± SD.

Immunohistochemistry

In brief, paraffin-embedded specimens cut into 5-µm sections and previously baked at 50°C for 30 min were twice deparaffinized 3x5min with xylene then rehydrated by immersion first with 100% ethanol for 5min, then 5min with 95% ethanol and finally 5min with 70% ethanol. When cell pellets from cell lines were used, the cells were first fixed with formalin overnight at room temperature, and then centrifuged at 3000 rpm for 10 minutes. After decanting the supernatant, the cell pellet was removed and wrapped in distilled moistened lens paper before the embedding process. Sections were washed 3x5min with PBS1X, submerged into hot (90°Celsius) 0,2M EDTA at pH 9 and microwaved 5min for antigen retrieval. After 15min of cooling at RT, sections were washed 3x5min with PBS and treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity. Following 3x5min wash with PBS, tissue sections were circled with a hydrophobic pen and rinsed to wash off excess hydrophobic ink. Approximately 150µl (enough volume to cover sections) of 2% BSA in PBS were used to block nonspecific binding for 30 min at RT. Approximately 150µl of primary antibodies diluted in blocking solution (enough volume to cover sections) were added to sections and incubated overnight at 4° Celsius.

Table 4. Summary of primary antibodies used for immunohistochemistry

Antibody	Catalog No	Dilution/ Concentration	Species/ Clonality	Antigen retrieval buffer*
LARP1	HPA054819 Sigma Aldrich	1:100	Rabbit IgG, Polyclonal	Na Citrate Ph 6.0
LARP1	Ab 86359 Abcam	1:100	Rabbit IgG, Polyclonal	Na Citrate Ph 6.0
LARP1	14763 Cell Signaling	1:100	Rabbit IgG, Polyclonal	Na Citrate Ph 6.0
LARP1	SC 102006 Santa Cruz	1:100	Rabbit IgG, Polyclonal	Na Citrate Ph 6.0
PAIP2	Ab33455 Abcam	1:100	Rabbit IgG, Monoclonal	Na Citrate Ph 6.0 and Tris-EDTA

As negative controls, equal amount of blocking solution without primary antibody was added to sections. PAIP2 antibody was used as positive primary antibody control. After washing 3x5min with PBS, the tissue sections were incubated with peroxidase-conjugated goat Anti Rabbit/Anti-Mouse IgG (1:100 in 2%BSA/PBS) for 1 h at 37^{o}C. The tissue sections were washed twice with PBS and approximately 150 µl (enough to cover sections) of DAB chromogen (20µl diluted in manufacturer's buffer) for visualization and counterstained with hematoxylin. After 5x3min washes with PBS, tissue sections were dehydrated with methanol cleaned with xylene and mounted in Acrytol mounting medium.

Cell growth and proliferation assay

CCK8 assay. The effect of LARP1 inhibition on cell proliferation was evaluated using cell counting kit-8 (CCK-8 kit, Dojindo, Japan). Scrambled (shSCR) and LARP1 silenced (shLARP1) cells were seeded into a 96-well plate at a density of 1500 cells in 100 µl DMEM containing 10% FBS and incubated at 37^{o}C with 5% CO2. 10 µl of CCK-8/well were added into each of the corresponding wells at the time intervals of 0, 24, 48, 72 and 96 h and continuously incubated at 37^{o}C with 5% CO2 for 1.5 h. The absorbance at 450 nm was recorded using a microplate reader for quantifying the relative cell numbers.

****Cell counting with Haemocytometer.*** To monitor cell growth, equal amount of scrambled (shSCR) and knockdown (shLARP1) cells were seeded into 24 well and cell number was calculated using an haemocytometer 24h, 48h, 72h and 96h after seeding. Counting was performed in quadruplicates, and results are presented as the mean ± SD.

In vitro cell migration assay

To compare the migration capacity of scrambled (shSCR) to that of the knockdown (shLARP1) cells were seeded into 12 well plates and cultured until 90% confluent. After wash with PBS, cells were scratched with 10 µl pipette tip. The pictures showing the reduction of the created distance between separated cells were taken right after the scratching (day0), 24h (day1), 48h (day2) and 72h (day3) later. The wound assay was performed in triplicate and the results analyzed with ImageJ are represented as the mean ± SD.

3. RESULTS

This study presents bioinformatics data resulting from different database where the mRNA and the protein expression of LARP1 and other genes (proteins) are compared in normal versus adjacent cancer tissues. These results also include cancer patients' survival data based on LARP1 expression and genetic alterations occurring with LARP1 in various cancers. These results were followed by in-vitro experiments meaning to validate selected commercially available LARP1 antibodies using three independent bioanalytical strategies. Moreover, further experiments determined the role of LARP1 in cell functions such as cell growth and cell proliferation indicating that LARP1 is evidently involved in the regulation of those functions. More analyses comparing parental cells and their LARP1-depleted adjacent cells indicated that LARP1 silencing may increase oncolytic viral infection in OVCAR8 cells and potentially alter the response to some cancer drugs.

Bioinformatics

Bioinformatics is an incredible tool for in-silico analyses of biological queries. Newly developed biological and computer methods have rendered possible the sequencing of many cancer genomes quickly and affordably. Given that adequate data reporting and annotation are

essential to ensure that results can be reproducible, assessment of in-silico databases can allow one to save time and effort in predicting the results of planned experiments. Here, I analyze the expression of LARP1 at mRNA level and protein level using different databases and established a correlation between LARP1 expression and cancer patient survival.

LARP1 expression and cancer patient survival (KMPLOT)

This bioinformatics study using the Kaplan-Meier plotter (www.kmplot.com) presents results indicating the correlation between cancer patient survival and the expression of LARP1. Different survival parameters were analyzed including overall survival (OS), progression free survival (PFS), distant metastasis free survival (DMFS) and regression free survival (RFS). Further analyses included some other genes such as those involved in the mTOR pathway and other genes known to be altered in various cancer such as BRCA1 in breast cancer.

Figure 6 presents the correlation between LARP1 expression level and the survival of breast, ovarian, lung and gastric cancer patients. Interestingly, breast cancer patients expressing high level of LARP1 (red curve) survived slightly worse than those with lower expression of LARP1 (black curve) (logrank P=0.005). Oppositely, gastric cancer patients with high expression of LARP1 survive much better than those with lower expression of LARP1 (logrank P<0.05). As for lung and ovarian cancer, there is neither clear nor strongly relevant correlation between LARP1 expression and patient survival (logrank P>0.05).

The analysis of certain genes involved in the wider mTOR pathway demonstrated some unexpected results. For example, the expression of genes with antagonistic functions correlates similarly with cancer patient survival (**Appendices _ Kmplot**). For example, high expression of both eIF4E1 and eIF4EBP1 correlates with poor prognosis among breast cancer patients while only the expression of eIF4E was expected to correlate with poor survival (**Appendices _ Kmplot**). Interestingly, Hopkins et al. [67] have previously claimed that high

expression level of LARP1 correlates with poor survival for ovarian cancer patients as the authors used the same dataset (www.kmplot.com) to support their claim. Surprisingly, my results demonstrate that there is no clear correlation between high expression of LARP1 and survival of ovarian cancer patients even when using the same parameters as described by the authors (number of patients, all stages and all grades included seen in **Figure 7)**. To further elucidate the correlation between LARP1 mRNA expression and ovarian cancer patient survival, I compared the results from different versions of the dataset (www.kmplot.com) including the previous and most recent versions of the dataset. Intriguingly, results presented by Hopkins and colleagues [67] could not be reproduced using all different available versions of the dataset (**Figure 7**).

Considering that the human LARP1b or LARP2 is a paralog of LARP1, I was interested in conducting the same analysis characterizing the survival of different breast cancer subtypes based on LARP1b mRNA expression. Notably, the overall survival results show that breast cancer patients with high expression of LARP1b have a better prognosis compared to those with lower LARP1 expression level (**Appendices _ Kmplot**).

Among the four cancer types analyzed, the results of the Kaplan-Meier analysis show that LARP1 may play a significant role in the survival of gastric cancer patients where high LARP1 expression correlates with better prognosis. It is, however, less clear at this stage whether such correlation could be established for other cancers analyzed with the Kaplan-Meier plotter. Unfortunately, the public version of the dataset (www.kmplot.com) does not offer survival data on prostate cancer patients for which LARP1 is suspected to be lost. I requested permission to use the private version which perhaps offers the survival data of more cancers including prostate cancer. Unfortunately, I did not receive any response from the developers (www.kmplot.com/private). Depending on the type of survival rate selected, the Kaplan-Meier curve can vary and exclude some patients in the study. To ensure that all

patients were correctly selected and represented in the analysis, the results from overall survival, progression free survival and post-progression survival were compared. However, my main analysis was based on the number of patients and the survival type recommended by the website for each of the four cancers studied (www.kmplot.com). Notably, results of different survival types coincide for ovarian cancer patients separated by LARP1 expression (**Appendices _ Kmplot**).

Surprisingly, when comparing LARP1 with some other genes known to be altered in specific cancers, it appears that even high expression of genes known as tumor suppressors or oncogenes for certain cancers does not distinctly correlate with better or poor prognosis respectively (**Appendices _ Kmplot**).

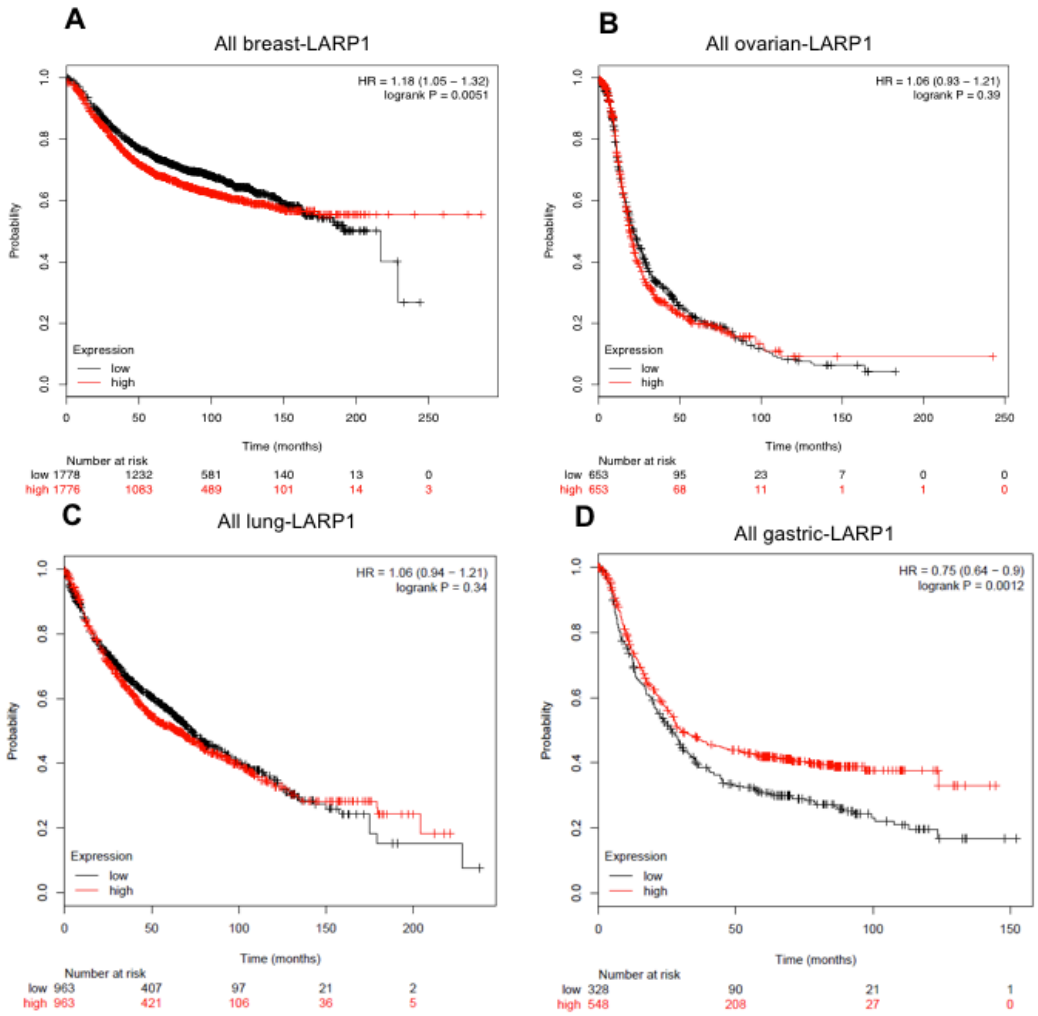

Figure 6. Kaplan-Meier analysis of Overall Survival in cancer patients. Breast cancer patients (A), ovarian cancer patients (B), lung cancer patients (C) and gastric cancer patients (D) were separated by LARP1 expression**.** Data Available at www.kmplot.com. Version 2015.

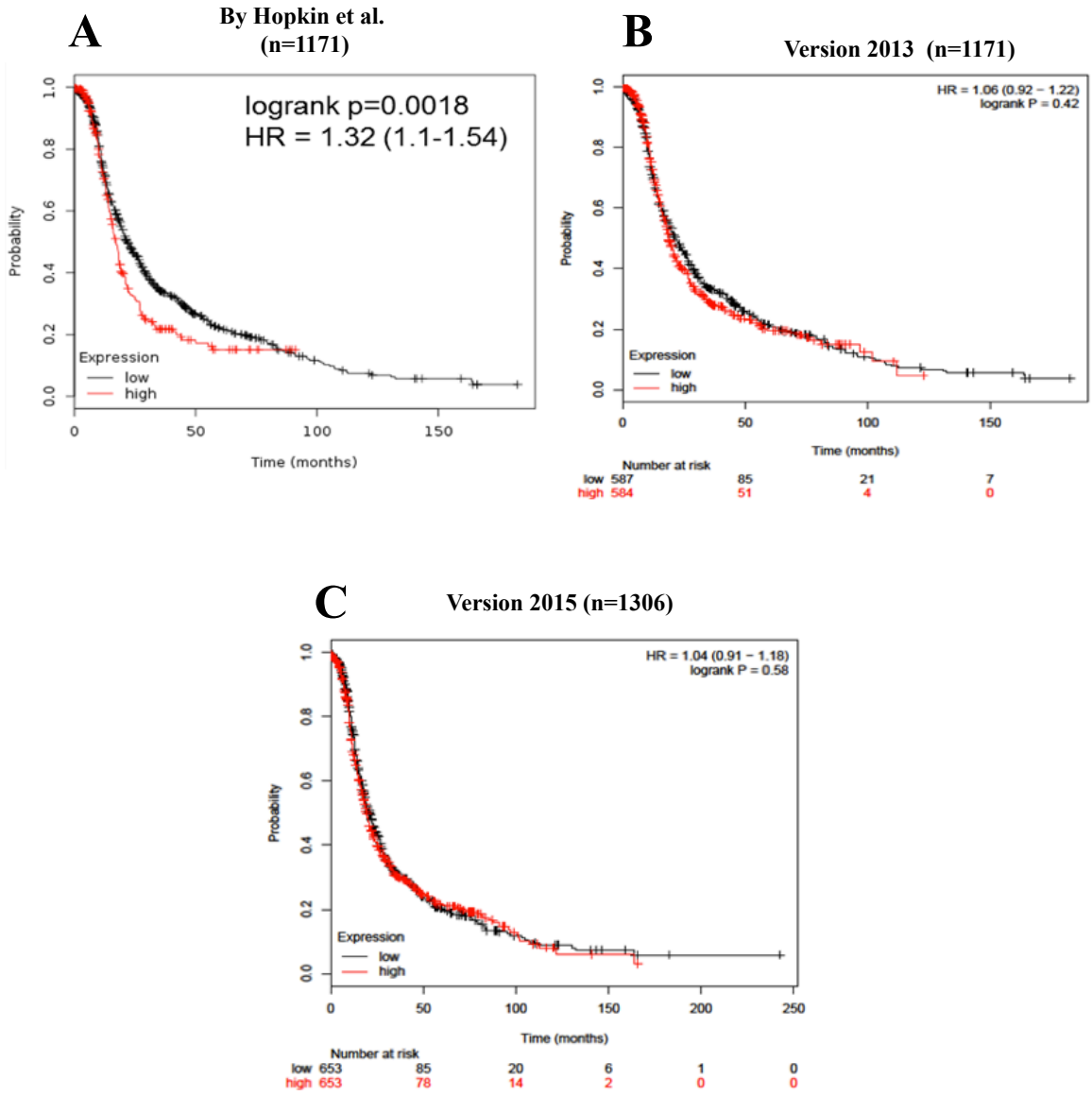

Figure 7. Kaplan-Meier analysis of PFS in ovarian cancer patients, separated by LARP1 expression. Results presented in Supplementary by Hopkins et al. (**A**) were not reproducible using the same version of the dataset (**B**). Results of the same analysis using a more recent version of kmplot.com (**C**). Data available at Kmplot.com

LARP1 mRNA expression in cancer cell lines (CCLE)

There is an ongoing discrepancy on the expression of LARP1 in cancer tissues, when compared to normal tissues. The claims supported by different groups on LARP1 expression in cancer are mainly based on comparison of different tissues and cell lines. Hopkins and colleagues [67] have shown that LARP1 is overexpressed in ovarian cancer tissues, while converging evidences from the Human Protein Atlas show that LARP1 is lost in prostate cancer tissues (www.proteinaltas.com). In this context, I interrogated the Cancer Cell Line Encyclopedia database (CCLE) (www.broadinstitute.org/ccle) in order to characterize the expression level of LARP1 in cancer cell lines grouped by corresponding tissue. Results were compared or confirmed by LARP1 mRNA expression level data from the Cancer Genome Atlas Database (TCGA; http://www.cbioportal.org). As expected, prostate cancer cell lines showed low expression level of LARP1 compared to other tumor cell lines. Chronic myeloid Leukemia, acute myeloid Leukemia cell lines and thyroid cell lines showed the highest expression of LARP1 compared with most of the tumor cell lines as seen in **Figure 8**. Surprisingly, more than fifty ovarian cancer cell lines included in the study did not show significantly elevated or decreased expression of LARP1 (**Figure 8**) as suggested by Blagden and colleagues [67]. The difference in expression level between normal tissues and adjacent cancer tissues is generally accepted as validation for biomarker discovery.

The Human Protein Atlas (www.proteinatlas.org) offers quantified expression level of genes for more than 25 different tissues from nearly 100 individuals. In the present study, the Human Protein Atlas database was queried in order to assess relative gene expression level of LARP1 in different human tissues. According to this database, brain and testis tissues remarkably express higher level of LARP1 compared to other tissues such as prostate, stomach, lung or liver where LARP1 seems to be less expressed (**Figure 9**).

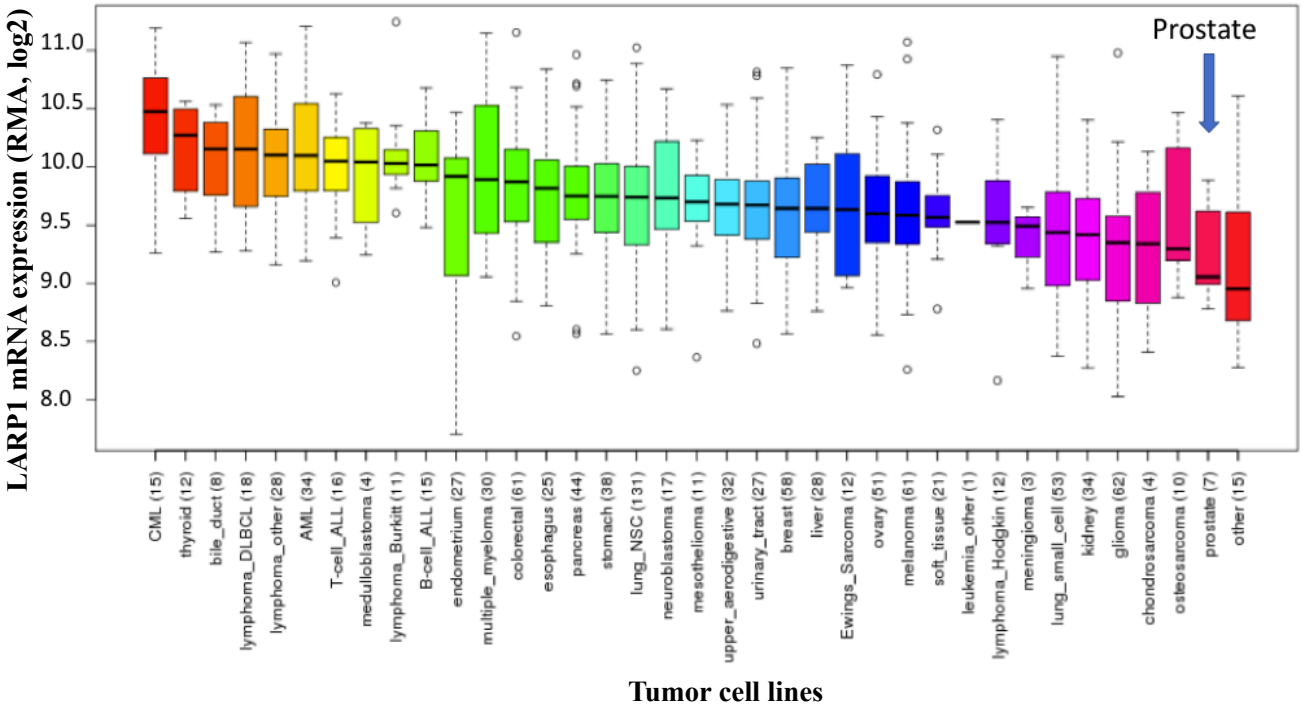

Figure 8. LARP1 mRNA expression in different tumor cell lines. Data generated from the cancer cell line encyclopedia (www.broadinstitute.org/ccle/home). Prostate cancer cell lines (indicated by blue arrow) show lower LARP1 expression compared to the other tumor types.

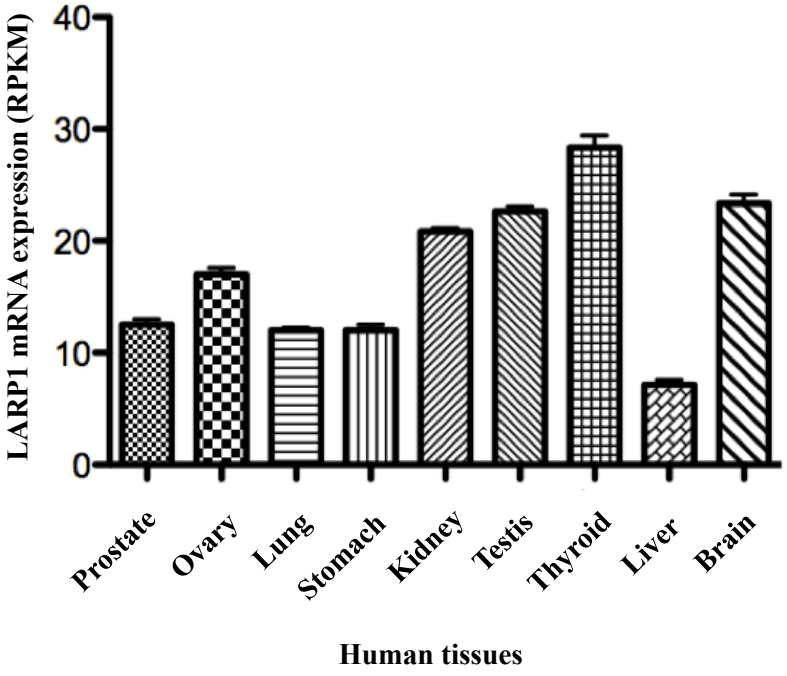

Figure 9. LARP1 mRNA expression in selected human tissues. Graphs were generated from the RNA-seq results of the Human Protein Atlas. 27 different tissues from 95 individuals were analyzed to determine tissue-specific gene expression.

LARP1 mRNA expression in cancer (ONCOMINE)

In the present study, the Oncomine database was queried to systematically characterize the relative gene expression levels of LARP1 and some other LARP1/mTOR- related genes in various tissues and in the tumor counterparts. Gene expression was compared between normal prostate, ovary, stomach, breast and lung versus their counterpart cancer tissues. From all results retrieved in Oncomine database when querying for LARP1 gene expression in normal tissue versus their tumor counterparts, only results with significant p-value ($P < 0.05$) were considered. To characterize the change in LARP1gene expression, fold changes between normal tissues versus cancer tissues from selected studies were compared. Some representative results of the analysis performed for LARP1 are represented in **Figure 10**. LARP1 is significantly upregulated in the Yoshihara et al. study [Yoshihara et al. _Oncomine] where ovarian tumor types against normal peritoneum are compared (fold change = 1.466, $P = 0.004$). While in the Chen et al. study [Chen et al._ Oncomine], LARP1 gene expression seems slightly higher in normal stomach compared to stomach tumors (fold change=1.264, $P = 2.49E-6$). The results from the Curtis et al. study showed that there is no significant difference in LARP1 gene expression between normal breast tissues and breast tumors (fold change =1.143, $P =0.024$). Furthermore, LARP1 gene expression in normal lung tissues is much lower than in lung cancer tissues based on results from the Steaman et al. study [Steaman et al _ Oncomine] (fold change = 1.565, $P = 8.10E-7$).

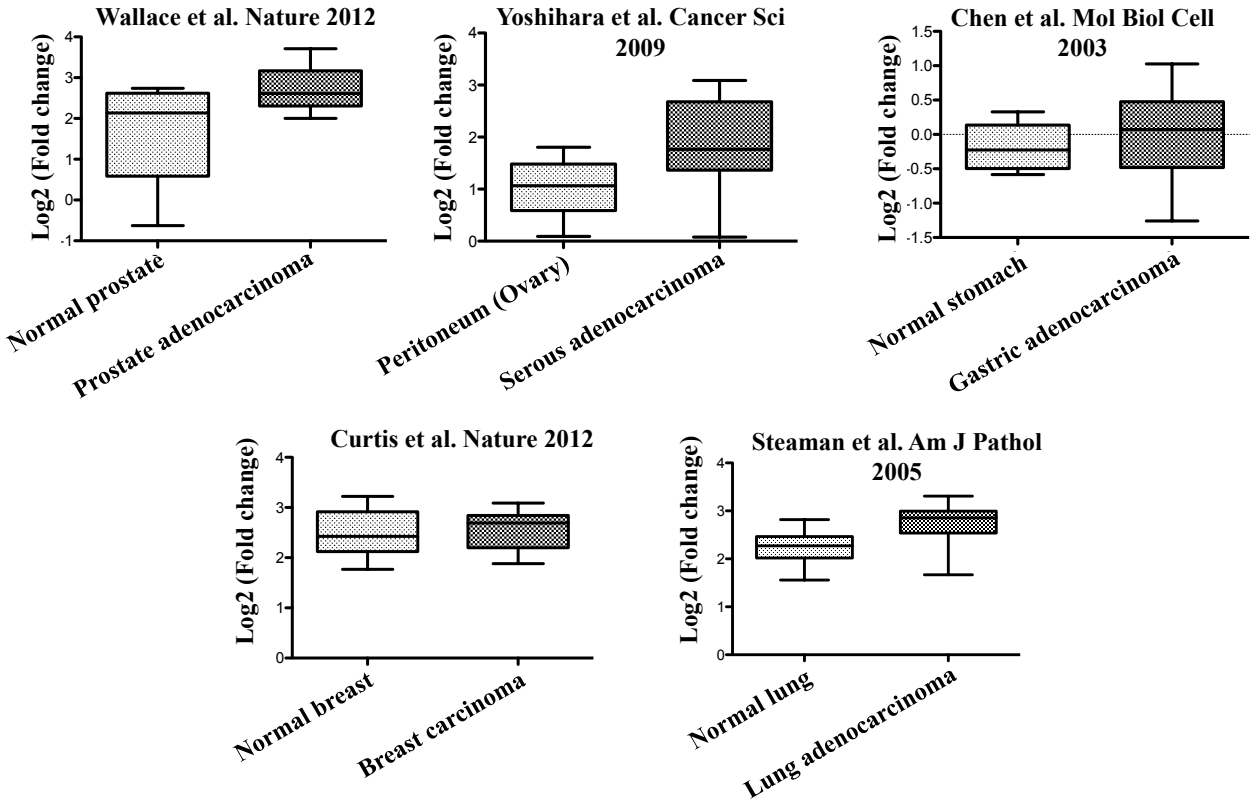

Figure 10. LARP1 expression in normal tissues and adjacent tumor tissues. Data from the same study are presented in single panel. Gene expression profile of LARP1 is presented in log2 transformed data. Data from Oncomine database (www.oncomine.org).

LARP1 genetic alterations in cancer (CBIOPORTAL)

The cBioportal website was used to evaluate molecular alterations occurring in LARP1 gene in normal versus cancer tissues or cell lines. The same analysis was performed for some other selected genes such as PTEN, MYC, MTOR and other LARPs when available. Furthermore, this comparison study of genetic alterations was conducted for other types of cancer including but not limited to breast cancer (Metabric study, n=2509 patients), lung cancer (TCGA provisional, n=522 patients), ovarian cancer (TCGA provisional, n=603 patients), all cancers combined by Pancancer studies (MSK-IMPACT clinical sequencing cohort, n=10946 samples) and various cancer cell lines (Cancer Cell Line Encyclopedia, n=1019 samples). For each cancer type, the study with the highest number of samples was preferentially selected if not otherwise mentioned. As genetic profiles, mutations and putative copy-number alterations were selected. Tumors with copy number alteration (CNA) data were selected in the query and the Human Genome Organization (HUGO) gene symbols were used to define the gene to be analyzed.

LARP1 demonstrated low somatic mutation, deletion and amplification rates for the majority of cancer studies available at Cbiorpotal. Even though previous studies have suggested a biomarker role for LARP1 in prostate cancer [26] and in ovarian cancer [67], LARP1 oncoprint (mean of visualizing distinct genomic alterations) results indicate that LARP1 is not significantly mutated in any cancer including ovarian and prostate cancer (**Figure 11**). However, the amplification frequency of LARP1 in neuroendocrine prostate cancer from the Trento and Cornell study was considerably higher (22%) than its amplification level in all other cancer studies (less than 8%) as seen in **Figure 12**.

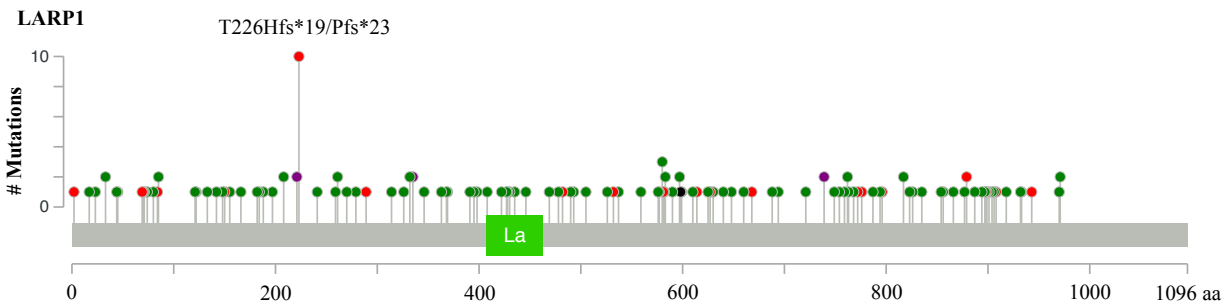

Figure 11. Representation of described LARP1 mutations occurring in cancer. Results for all cancer studies combined. The highest number of mutations with location within the amino acid sequence is indicated. Missense mutations are represented by green circles and truncating mutations are represented by black circles, inframe mutations are represented by red circles and all other types of mutations are represented by purple circles. Data from the Cancer Genome Atlas Cbioportal.

To further elucidate LARP1 alteration in Neuroendocrine Prostate cancer (NEPC), a comparative quantification study of gene alterations between LARP1 and some other selected genes was performed. PTEN and MYC were added to comparison as well-known tumor-suppressor and oncogene respectively by their expression and genetic alteration in most cancers including prostate cancer. BRCA1 and AR genes were also included in the comparison as they are known to be altered in most epithelial cancers such as breast cancer.

The genetic alterations of other members of the LA-related proteins were also compared to alterations occurring with LARP1 in different cancers when available. Interestingly, the results suggest that LARP1 is genetically altered as much as PTEN (31%) and far much more than MTOR (14%), BRCA1 (22%) and all other LARPs (2-12%) in NEPC patients (**Figure 13**).

Network analysis to predict LARP1interactome

Network view of genes expected to interact with LARP1 is presented in **Figure 14**. As expected, several genes involved in the mTOR pathway and a plethora of genes coding for ribosomal proteins are part of the LARP1 interactome. Interestingly, LARP1 is also predicted to interact with Lin28A, a microRNA known to be involved in cancer progression [69].

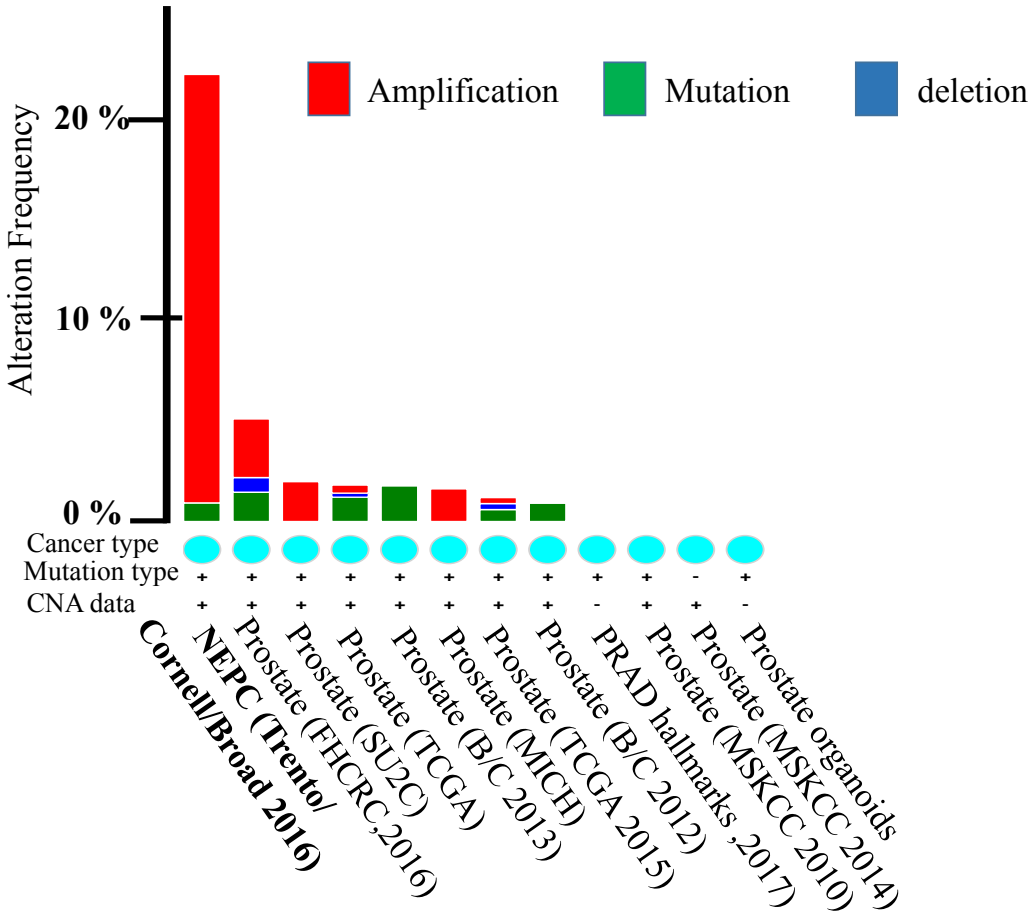

Figure 12. Mutation diagram of LARP1 in different prostate cancer types. LARP1 mutation frequencies (mainly amplification) are the highest in the neuroendocrine prostate cancer compared to other prostate cancers. Red bars represent amplification frequency, green bars represent mutation frequency, and blue bars represent deletion frequency. Data from The Cancer Genome Atlas Cbioportal.

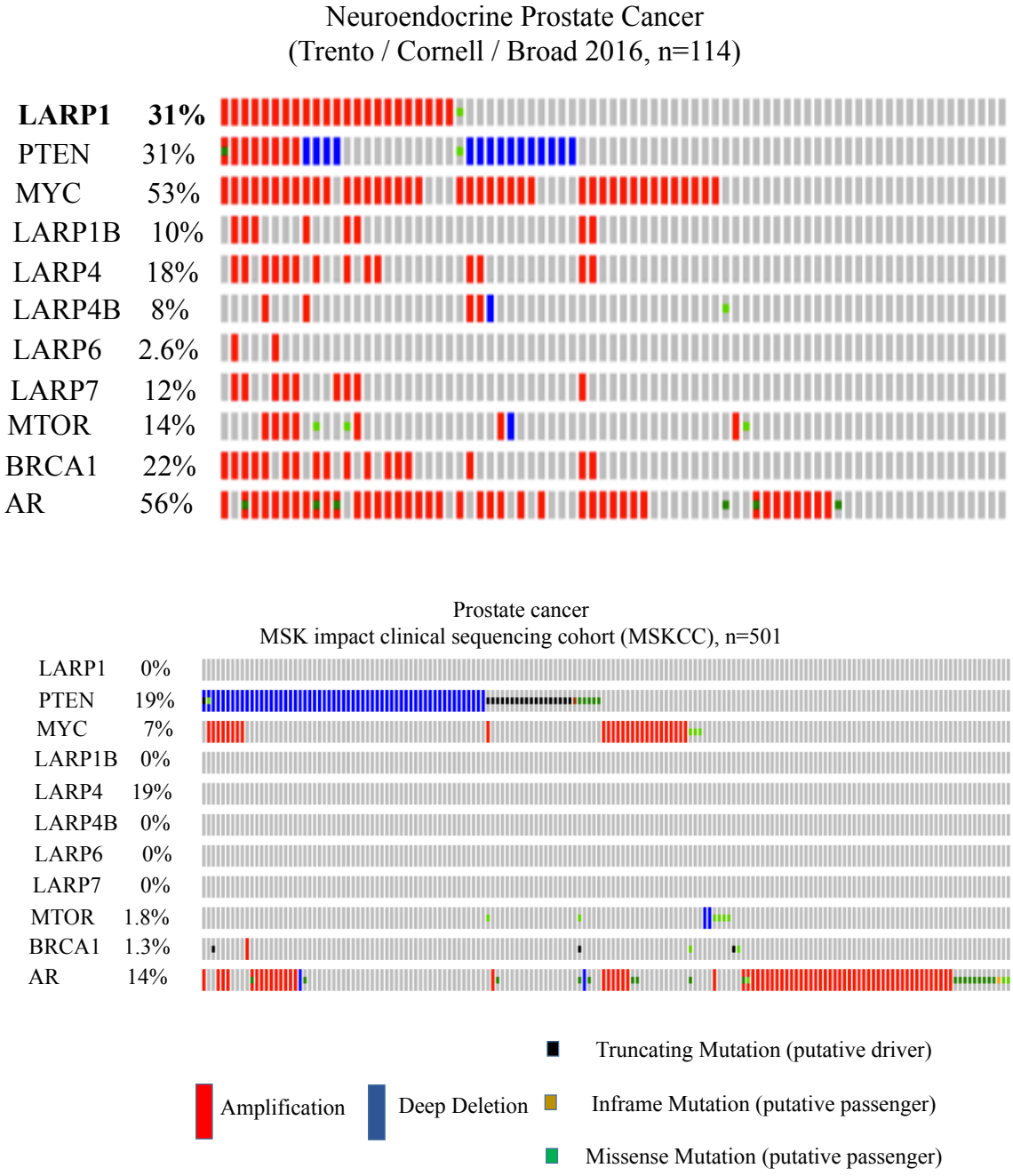

Figure 13. Genetic alteration frequency of LARP1 and other selected genes in three different prostate cancers studies. Grey bars along a vertical line represent the same sample interrogated for amplification (red), deep deletion (blue), missense mutation (green), truncating mutation (black) or in-frame mutation (brown). Data from The Cancer Genome Atlas Cbioportal.

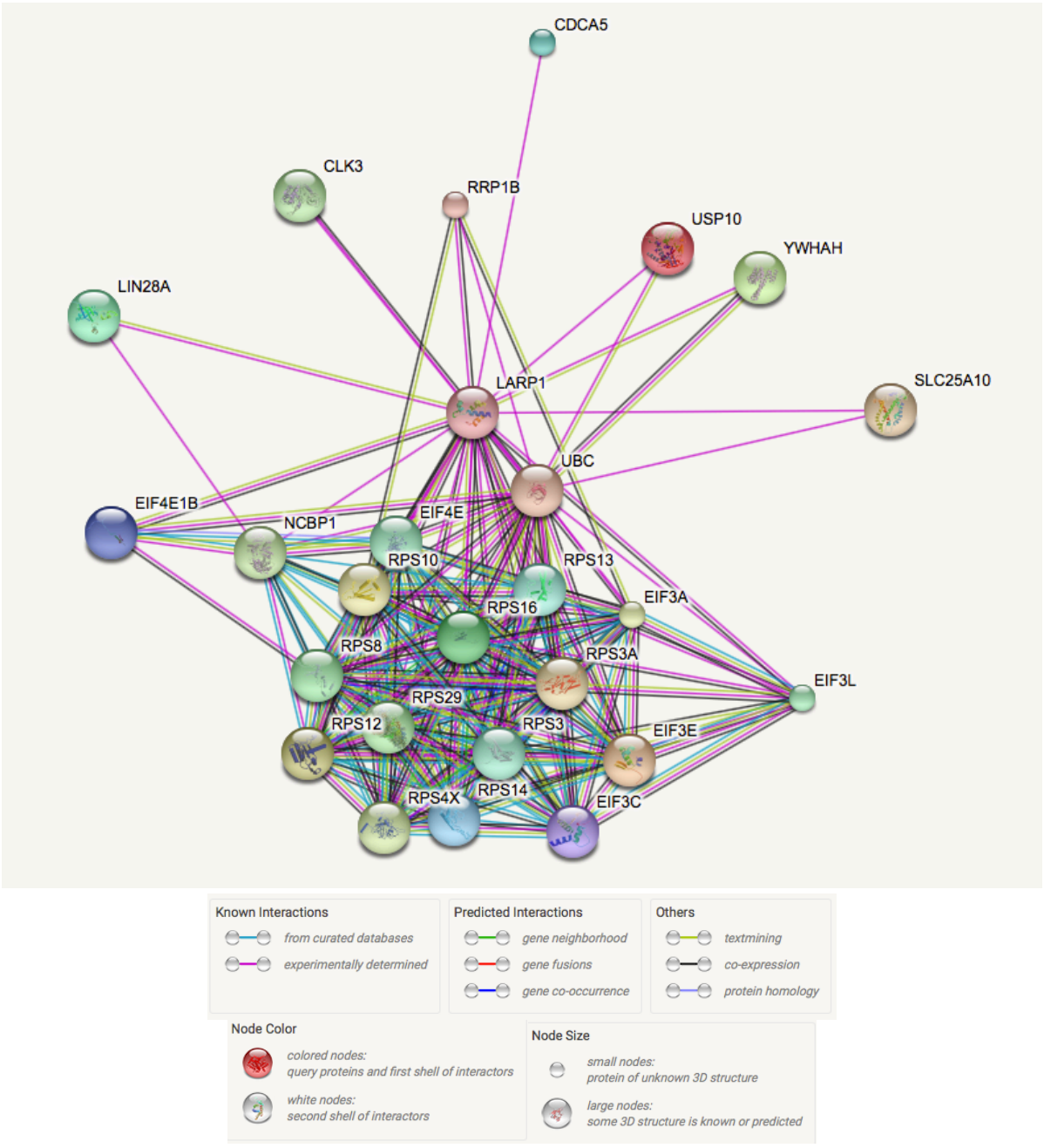

Figure 14. Network analysis to predict LARP1 interactome using a network prediction tool. The predicted associations are derived from the mining of databases and literature, from high-throughput experimental data and from other computational predictions based on genomic analysis. Data from STRING database (www.string-db.org).

Analysis of LARP1 protein expression in cancer progression

The Human Protein Atlas

To further investigate the functional significance of LARP1 expression in cancer progression, this study included comparing the protein expression level of LARP1 in normal tissues versus their corresponding cancer tissues from patients. Tissue microarray pictures of normal and cancer tissues (prostate, stomach, ovary, lung, kidney, breast) were systematically characterized and compared based on LARP1 expression. Furthermore, the protein expression of other proteins of the mTOR pathway, LARP1b and others were also analyzed. In total, more than 4000 pictures were screened and classified by cancer type, by antibody used for immunohistochemistry staining and by protein expression. Patient information such as age and gender are also presented. **Figure 15** gives an overview of selected normal tissues compared to adjacent cancer tissues.

Remarkably, the results of this comparison demonstrated that LARP1 is highly expressed in the glandular cells of normal prostate tissue (**Figure 15-A**) and in normal stomach tissue (**Figure 15-B),** while considerably lost in their corresponding cancer tissues counterparts. Analysis of single female patients demonstrated that LARP1 expression seems lower in ovarian cancer tissues compared to normal ovary of selected tissue microarray (**Figure 15-C).** There is no clear conclusion to be made for breast cancer tissues (**Figure 15-D**), kidney cancer tissues (**Figure 15-E**) and lung cancer tissues (**Figure 15-F**), where both normal and cancer tissues seem to similarly express LARP1. An overall view of all cancer patients separated by LARP1 expression confirmed a loss of expression in prostate cancer (**Figure 16**) and gastric cancer (**Figure 17**) while no major difference was observed between normal ovarian tissues and adjacent ovarian cancer tumors (**Figure 18**).

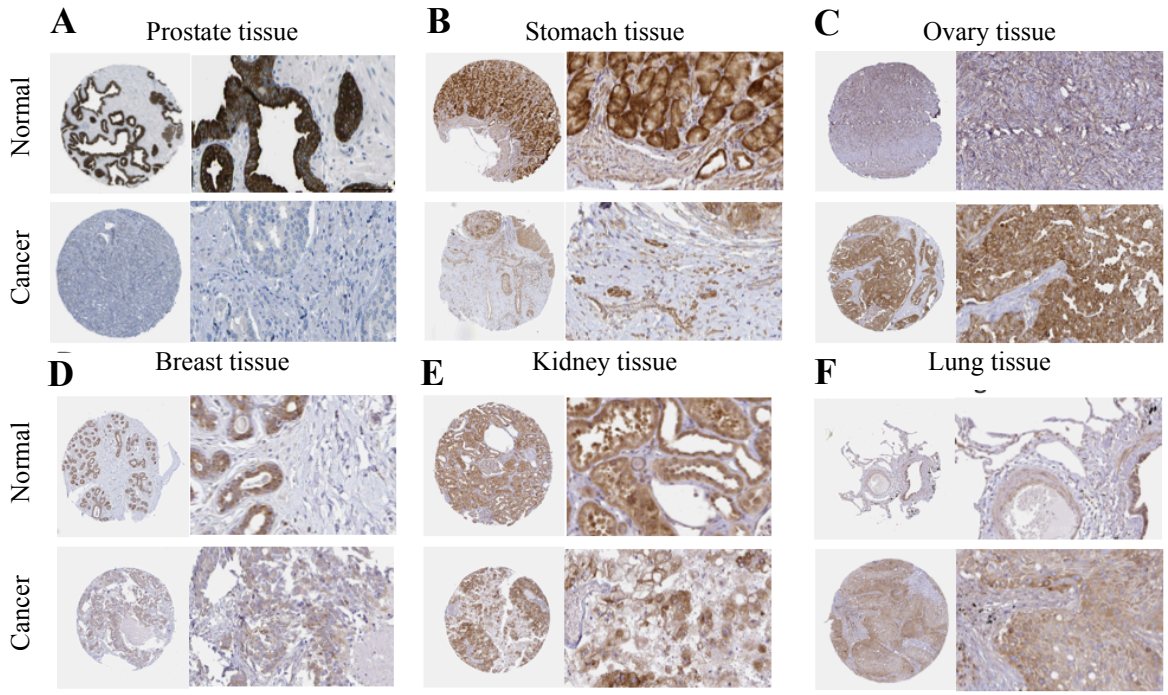

Figure 15. Representative tissue microarray (TMA) pictures of LARP1 protein expression in cancer. Normal prostate (**A**), stomach (**B**), Ovarian (**C**), breast (**D**), kidney (**E**) and lung (**F**) tissues are compared to the adjacent cancer tissues. Staining with CAB015222 antibody (prostate) and HPA051397 antibody (Stomach, ovarian, breast, kidney and lung). Data extracted and assembled from www.proteinatlas.org.

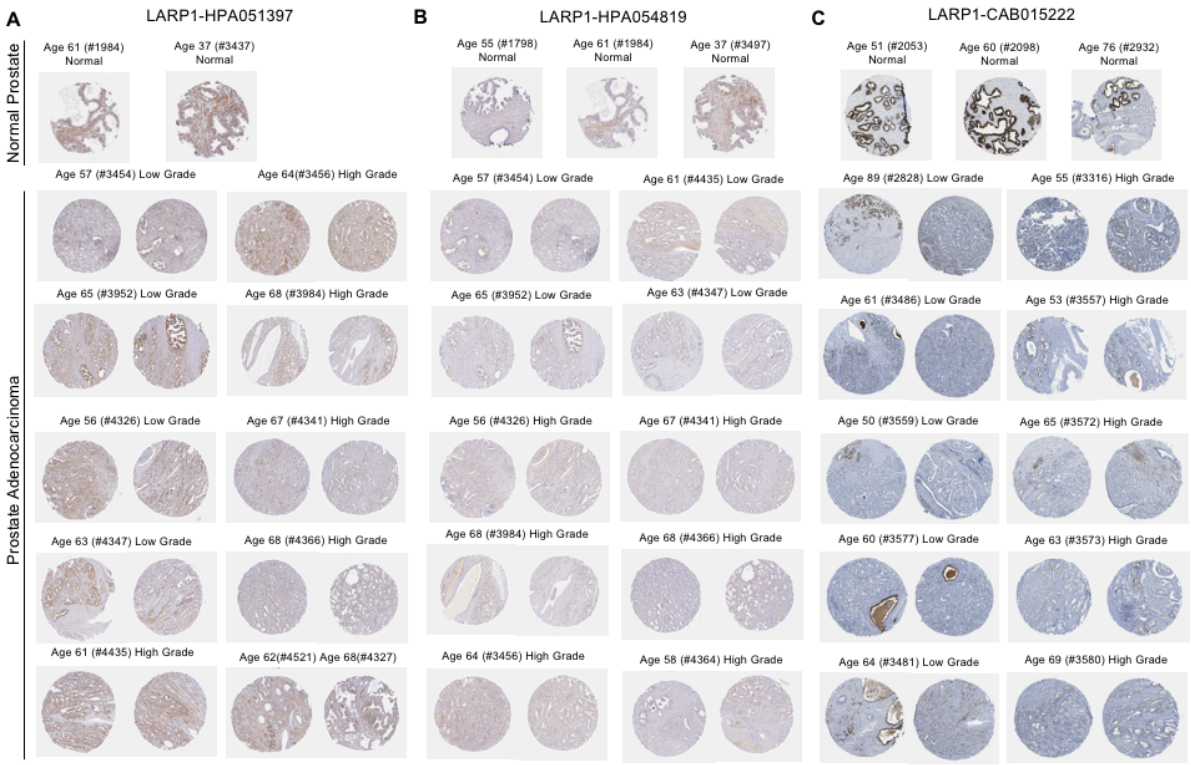

Figure 16. Representative pictures of LARP1 protein expression in normal prostate and prostate cancer tissues with tissue microarray (TMA). A: staining with HPA051397 antibody. B: staining with HPA054819 antibody. C: staining with CAB015222 antibody. Data extracted and assembled from www.proteinatlas.org.

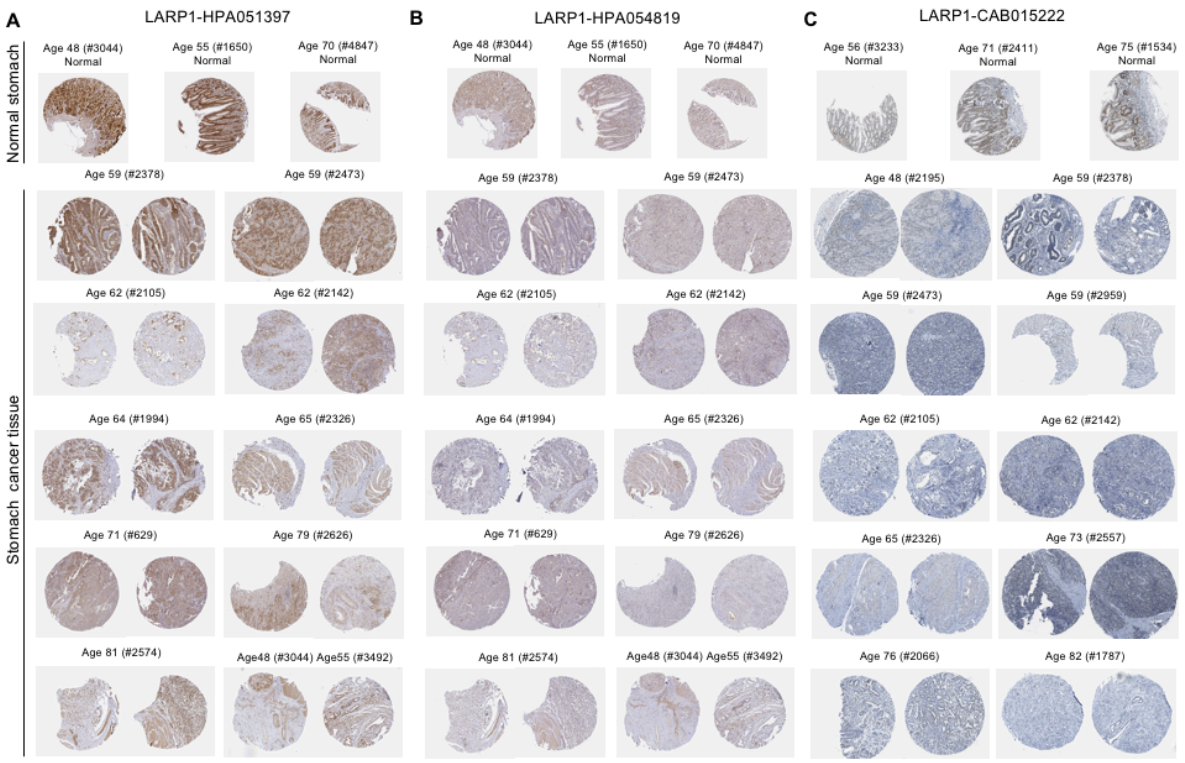

Figure 17. Representative pictures of LARP1 protein expression in normal stomach and stomach cancer tissues with tissue microarray (TMA). A: staining with HPA051397 antibody. B: staining with HPA054819 antibody. C: staining with CAB015222 antibody. Data extracted and assembled from www.proteinatlas.org.

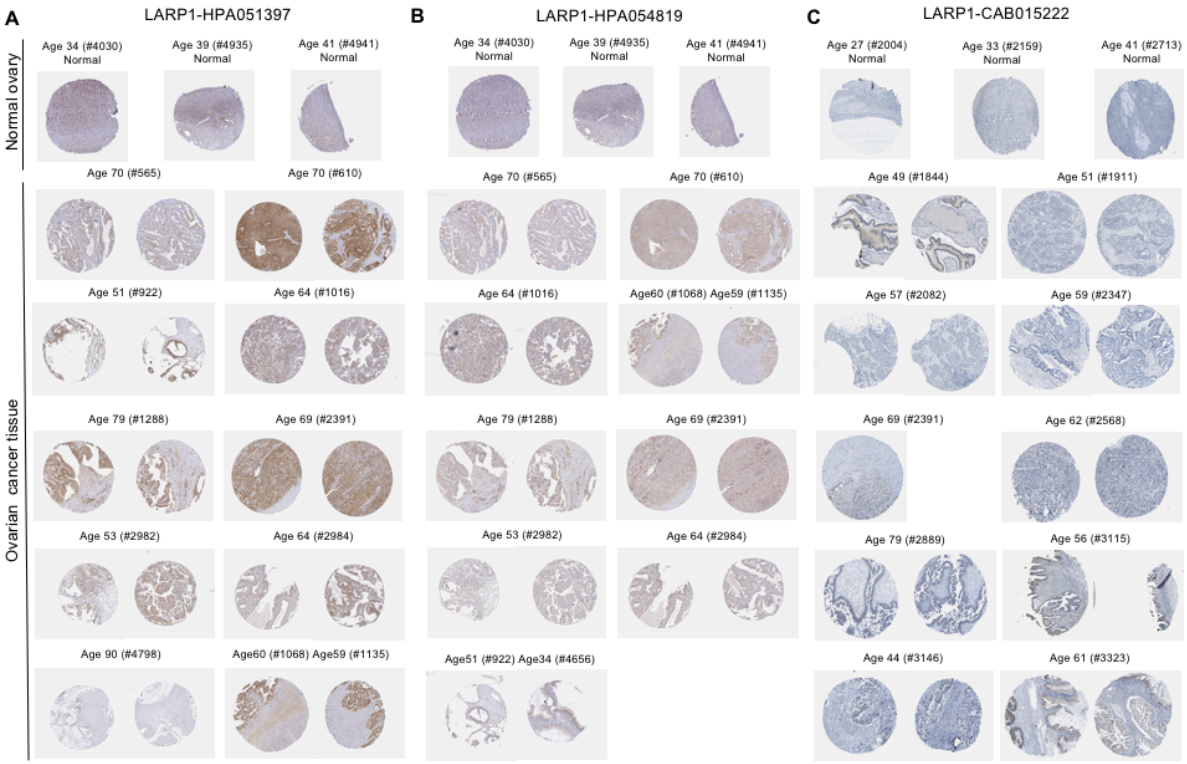

Figure 18. Representative pictures of LARP1 protein expression in normal ovary and ovarian cancer tissues with tissue microarray (TMA). A: staining with HPA051397 antibody. B: staining with HPA054819 antibody. C: staining with CAB015222 antibody. Data extracted and assembled from www.proteinatlas.org.

Overall views of different protein expressions for all patients available are also presented in **Appendices** for each cancer and each protein separately (**Appendices _ Human Protein Atlas**).

Out of the three LARP1 antibodies used for the immunochemistry staining, two demonstrated similar staining pattern (HPA051397 and HPA 0548919). However, the first (HPA051397) stained more strongly than the second (HPA054819) in all tissues analyzed. The third LARP1 antibody (CAB015022) stained strongly in prostate and gastric tissues and very poorly in breast and lung tissues. Taken together, the results from the Human Protein Atlas suggest that LARP1 expression is lost in prostate and gastric cancer. This observation leads me to believe that LARP1 is a potential tumor suppressor for these two cancers mentioned above. Nevertheless, the quality of the staining and the reliability of the antibodies used represent a considerable issue for interpreting the tissue microarray results, especially for the other cancers such as ovarian and lung cancer.

Cell culture

In order to study the specific role of LARP1 in cancer progression and to validate commercially antibodies for LARP1 detection, LARP1 expression was silenced in different cancer cell lines using lentivirus transfection. Cells were selected in puromycin for five days and parental cells were used as negative control (**Figure 19**). The effect of puromycin on selected cells was monitored and captured with electronical microscope. Only cells successfully transduced by lentiviruses survived (sh scrambled and shLARP1), and parental cells (not transduced by the lentivirus) were used as negative controls.

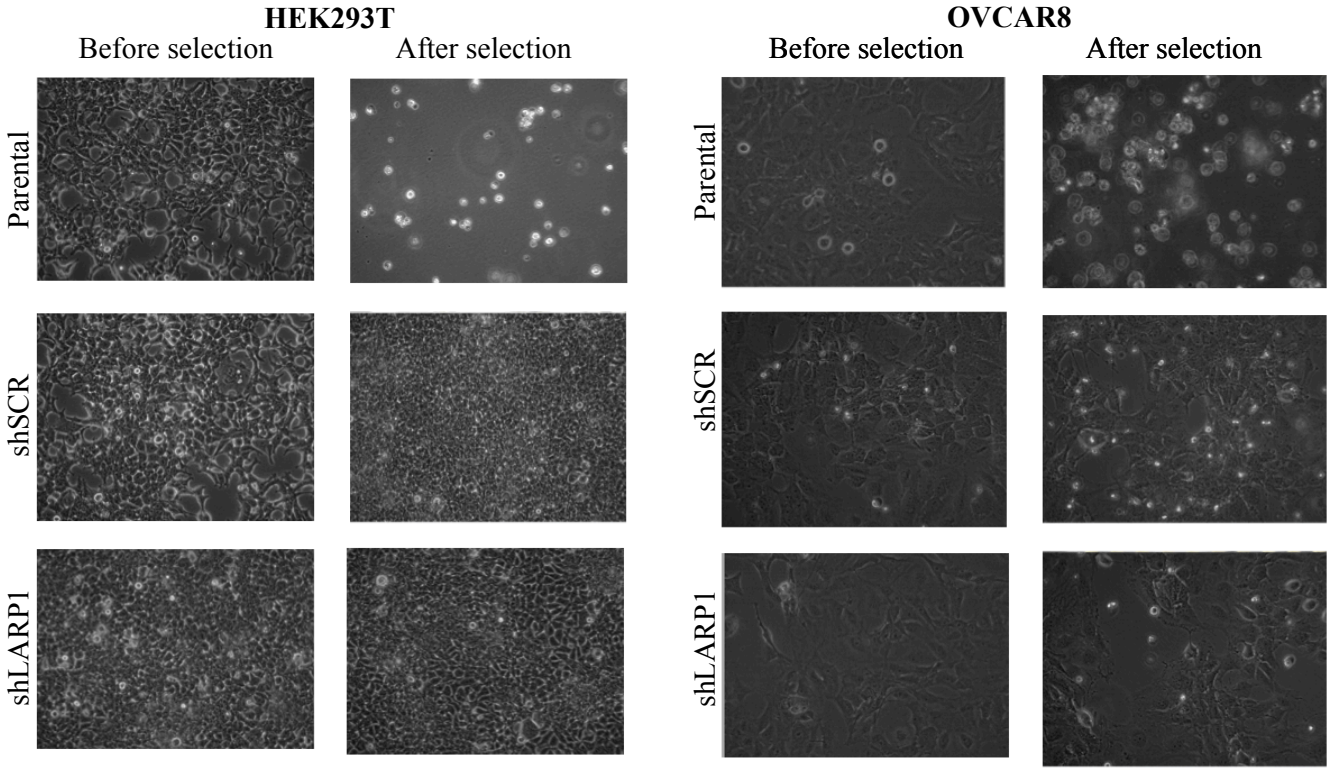

Figure 19. Selection of stably transduced HEK293T (left) and OVCAR8 (right) cells using puromycin. Cells were treated with puromycin for 5 days. Parental cells not transduced by lentiviruses (rounded cells) were washed away with PBS. Only successfully transduced puromycin resistant cells (shLARP1 and shSCR) survived.

Validation of LARP1 antibodies

It's well known that the choice of reagent is crucial when performing assays whose results might be used to develop or improve therapeutic strategies for cancer patients. Depending on the reliability of the reagents selected for the experiments, results can be biased, unrepeatable or unusable. Such consideration requires the selection of validated and highly specific antibodies for immunoassays where potential biomarkers are screened. Although the use of antibodies is a routine in basic research science and in clinical immunoassays, there is unequivocally a real need of validating antibodies that are included in bioanalytical assays of patient samples; especially for newly described proteins.

When analyzing data from the Human Protein Atlas, it appeared that two of the three antibodies used to stain for LARP1 were not validated; surprisingly two of these antibodies were deemed "unreliable" and the other one "uncertain" (**Figure 20**). This information, available on the Human Protein Atlas project web site (www.proteinatlas.org) proves in a convincing manner, how rigorously and fastidiously reagents should be selected when planning experiments. Considering the facts cited above, rigorous selection and validation of commercially available LARP1 antibodies soon became a necessity. In order to do so, three widely used techniques were selected to stain for LARP1 in scrambled (shSCR) in knockdown cells (shLARP1) with different LARP1 antibodies: Western blotting, Immunofluorescence and eventually Immunohistochemistry. Specificity of the antibody would be confirmed by the absence of staining in knockdown cells, where LARP1 has been silenced.

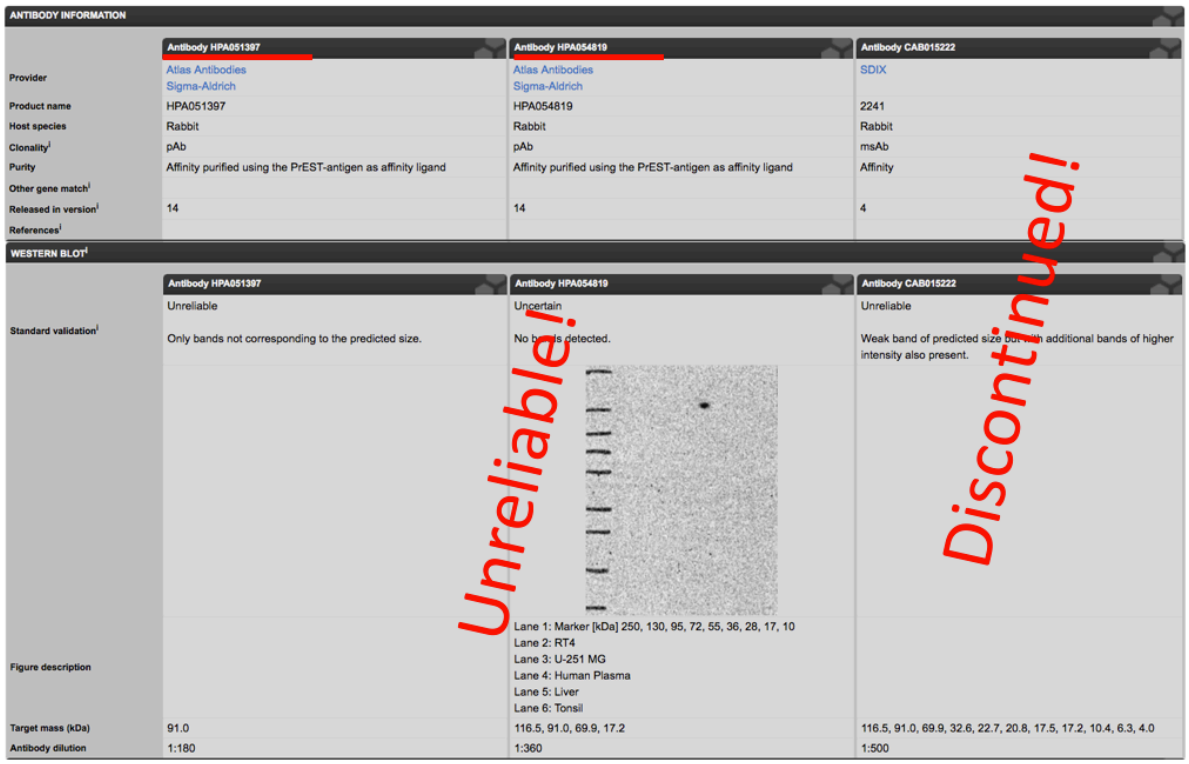

Figure 20. Validation of anti- LARP1 antibodies used in the Human Protein Atlas. Out of the three LARP1 antibodies used, none was validated by Western blotting. The third antibody (CAB15022) is discontinued. Screen capture from the Human Protein Atlas web page (www.proteinatlas.org).

Antibody validation by Western blotting

In the present study, Western blots were used to compare the specificity of four different LARP1 antibodies, used at the exact same conditions. The results show that the LARP1 antibody from Santa Cruz (SC 102006) is less specific and stains less strongly compared to the ones from Sigma, Abcam and Cell Signaling for HEK293T and OVCAR8 cells (**Figure 21**). Moreover, when tested on mouse cancer cell lines, even LARP1 antibody from Sigma did not stain as specifically and strongly as for human cell lines (HEK293T and OVCAR8). The representative results for the CT2A mouse glioma cell line presented in **Figure 22** showed that the LARP1 antibodies tested do not specifically detect mouse Larp1. However, it's not excluded that further optimization of the protocol used in this study could improve the staining quality for mouse tissues. Nonetheless, other mouse cell lines (such as 4T1) should also be tested to ensure that the results observed for CT2A are not specific for those cells (For example, CT2A cells may not produce LARP1). Together, the results presented above demonstrate that most LARP1 antibodies stain relatively well in human cell lines, but poorly detect mouse LARP1 in a murine cell line.

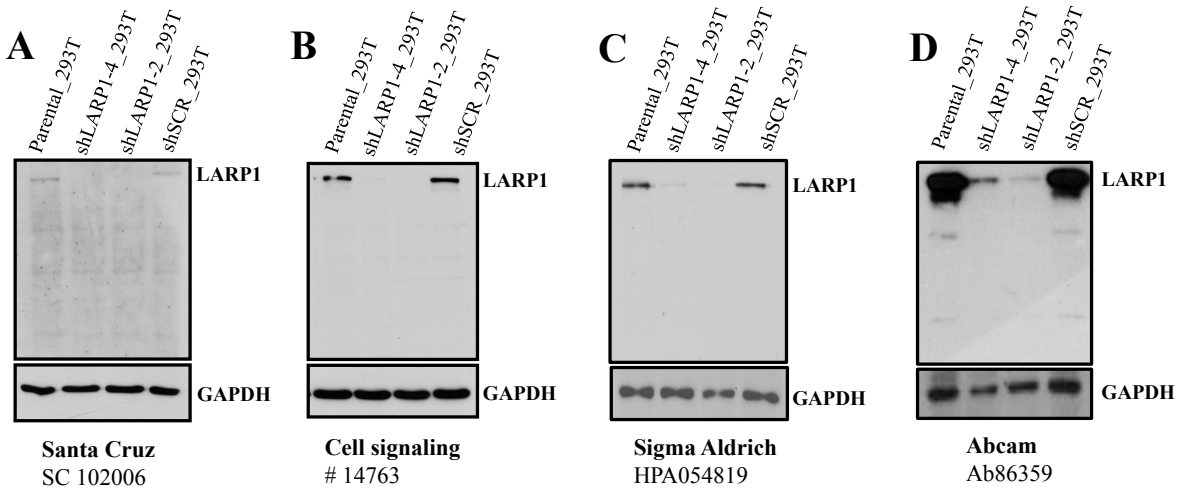

Figure 21. Validation of selected commercially available LARP1 antibodies by Western blotting. HEK293T cells were transfected with indicated plasmids and selected on puromycin for 5 days. Cell lysates were subjected to Western blotting. LARP1 antibody from Santa Cruz (**A**), Cell Signaling (**B**), Sigma Aldrich (**C**) and Abcam (**D**) were compared using the same conditions in the materials and methods section.

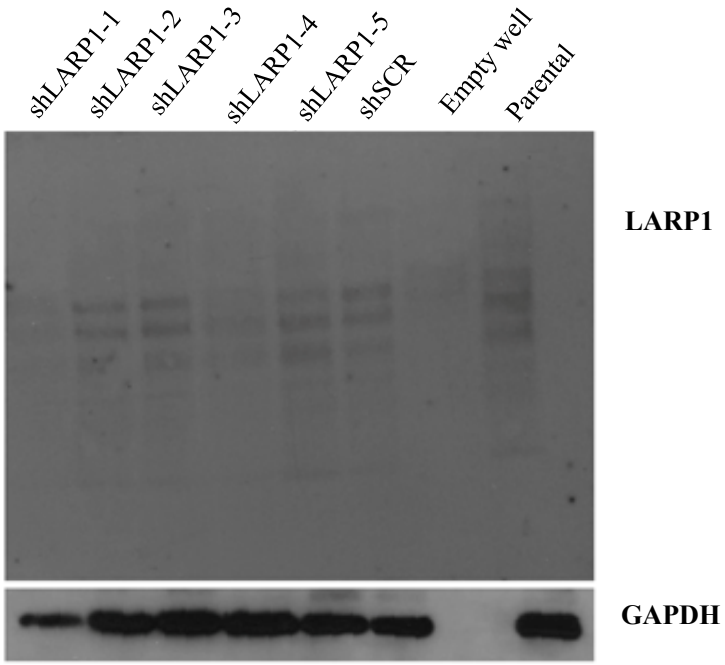

Figure 22. LARP1 expression in mouse cell line (CT2A) using LARP1 antibody from Sigma (HPA 054819). No specific bands were observed for scrambled (shSCR) and parental (wild type) as well as for the Knockdown (shLARP1s) cells. 5 different shRNAs targeting LARP1 were tested (1-5).

Antibody validation by Immunofluorescence

Western blotting is a good first step to validate antibodies, but surely not the most efficient neither the absolute method for antibody validation. Considering the structural conformation of proteins, one should consider using more than one method to validate antibodies; especially when antibodies will be used for other immunoassays such as immunofluorescence or immunohistochemistry.

One of the main challenges when selecting antibodies is to demonstrate that the latter selected is able to recognize in a specific manner the target when used for various applications: when a protein is in its native –structural conformation as well as when fully denatured during a SDS-PAGE run. For that purpose, selected LARP1 antibodies were validated using immunofluorescence, by quantifying and comparing the expression of LARP1 in scrambled (shSCR) and in knockdown cells (shLARP1). The results showed that LARP1 antibodies from Sigma and Abcam stained much more specifically and more strongly than those from Santa Cruz and Cell Signaling (**Figure 23**).

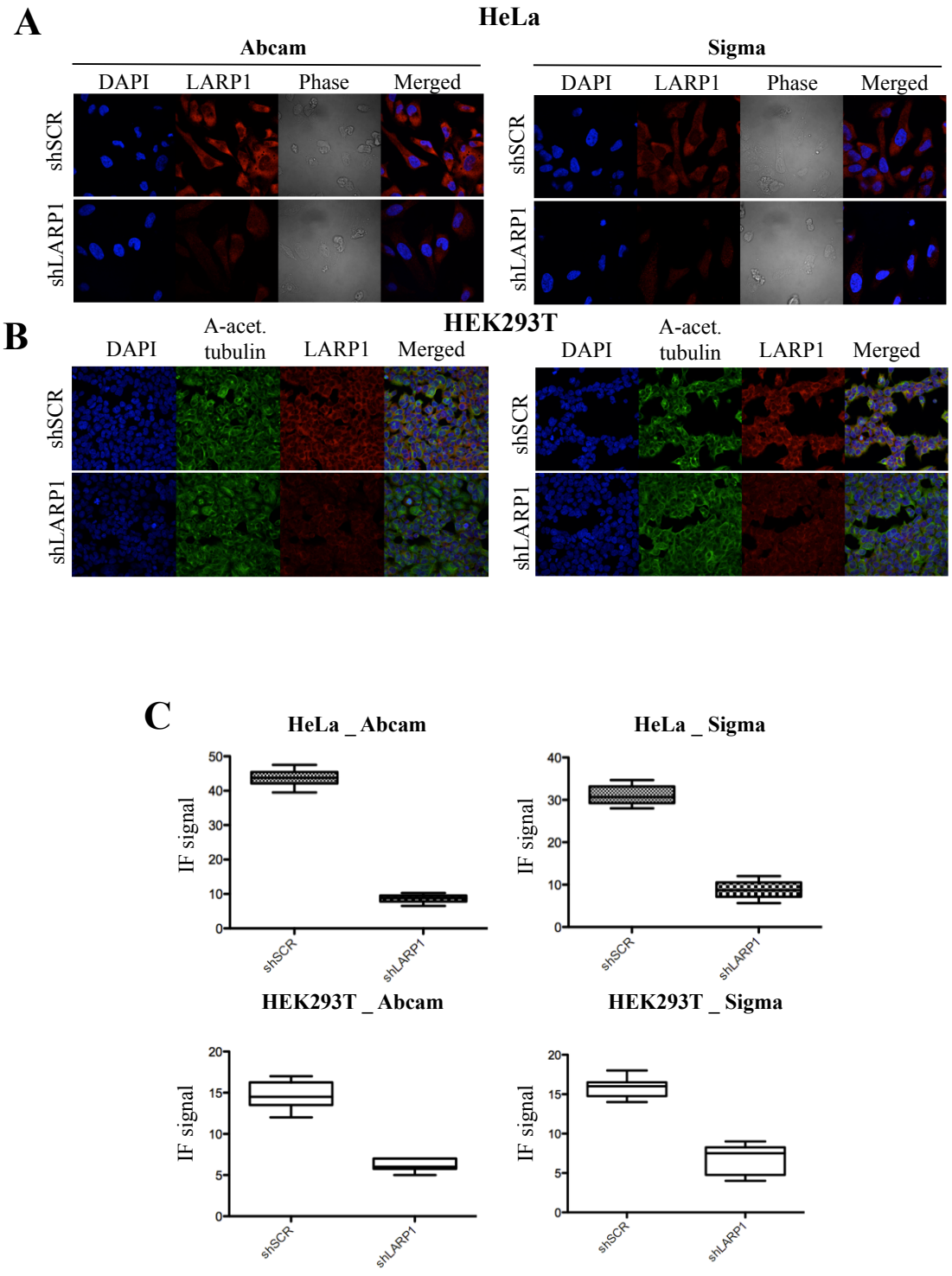

Figure 23. Validation of selected LARP1 antibodies by immunofluorescence. HeLa cells (A) and HEK293T cells (B) were transfected with indicated plasmids using lentivirus system and selected on puromycin for five days. LARP1 expression of at least 5 selected confocal cell images was quantified as "IF signal" using image J and Prism software.

Antibody validation by Immunohistochemistry

To clearly and unequivocally validate the selected LARP1 antibodies, LARP1 expression was compared between normal and cancer tissues using Immunohistochemitry. Unfortunately, I was not able to obtain tumor and adjacent normal tissues from the same patient. Moreover, there is no way to obtain human tissues with silenced LARP1 which could be compared to "normal adjacent" human tissues of the same organ. I therefore decided to use cell pellets of human OVCAR8 cells from both scrambled (shSCR) and knockdown (shLARP1) cells. This strategy would allow me to confirm the specificity of selected LARP1 antibodies used for the immunohistochemistry experiments. Next, LARP1 expression was assessed in the various human cancer tissues which were available to us, as well as use different LARP1 antibodies in mouse tissues. This is based on previous results showing that LARP1 is highly expressed in mouse testis and brain (www.proteinatlas.com).

As seen in **Figure 24**, the overall results of the validation by immunohistochemistry showed a poor and non-specific staining. Most strikingly, the counterstaining with hematoxylin is almost not visible in the IHC pictures of OVCAR8 cell block (**Figure 24**). This can be explained by a higher concentration of the antibody or simply by nonspecific binding. Also, the fact that cell blocks were stained as pellet and fixed in formalin can explain a possible nuclear smear which leads to unspecific staining. Cell blocks are cell pellet (in our case) or microbiopsies (in general) processed and embedded in paraffin used in cytology or histopathology to broaden the diagnostic value of cancer patients. In general, the samples for cell blocks are obtained from urine, effusion fluids, sputum or by fine needle aspiration cytology (FNAC). The samples from normal tissues are then compared with adjacent tumors. The only reason cell blocks were used in this study was because of the difficulty to obtain human normal tissues and their adjacent tumor tissues from the same patient. Nonetheless, there is a slight difference in LARP1 staining (brown) between scrambled cells and

knockdown cells. This is even more visible for the Cell Signaling LARP1 antibody (**Figure 24**).

As expected from my Western blots, most LARP1 antibodies used to stain in mouse tissues demonstrated a poor and non-specific binding. The rationale of this experiment was to compare the staining of mouse tissues with human tonsils, considered as a "positive control". Tonsil tissue is well accepted by many as a positive control in the immunohistochemistry field[2]; LARP1 is found to be highly expressed in the testis (www.proteinatlas.com). The antibody from Abcam shows a strong and non-specific binding while the antibodies from Cell Signaling, Santa Cruz and Protein Tech did not stain mouse tissues at all (**Figure 25**).

Therefore, the results demonstrate that the commercial LARP1 antibodies might not be optimal for all applications (good staining by Western blot, but weak staining for IF or IHC), and should be systematically validated for the cell lines or tissues analyzed based on the selected application.

[2] https://histologistics.com/histology/recommended-ihc-controls/

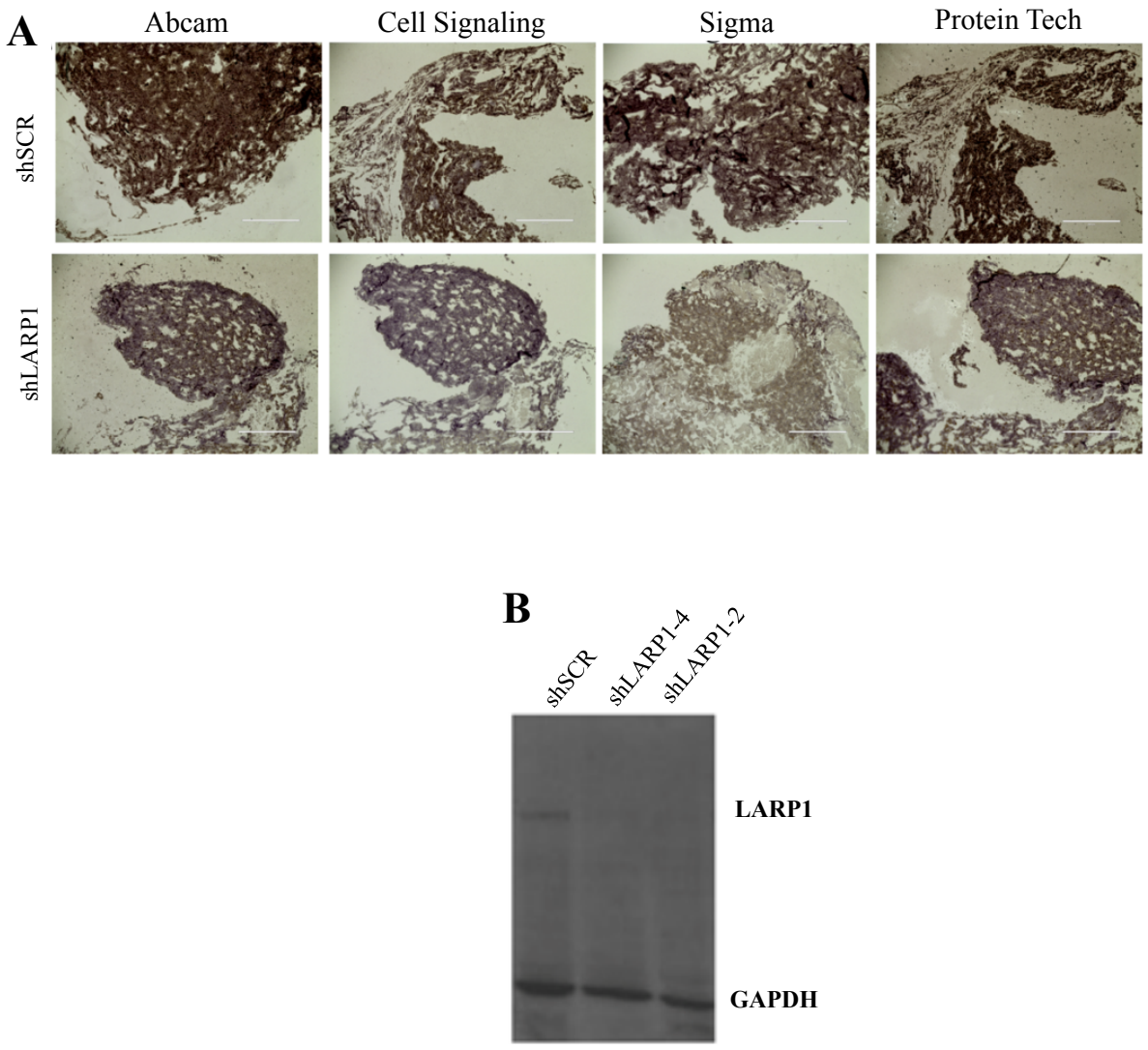

Figure 24. LARP1 antibody validation by immunohistochemistry. (**A)** Selected commercially available antibodies targeting the LARP1 protein at different epitopes were tested using immunohistochemistry on cell pellet fixed with paraffin. Staining examples from OVCAR8 scrambled cells (sh SCR) and LARP1-silenced OVCAR8 cell pellet (shLARP1) are shown. Chromogen 3, 3'-Diaminobenzidine (DAB) was used for the staining. Cells were counterstained with hematoxylin. (**B)** Western blot analysis of LARP1 expression in OVCAR8 cell pellet. All LARP1 antibodies were used at 1:50 dilution.

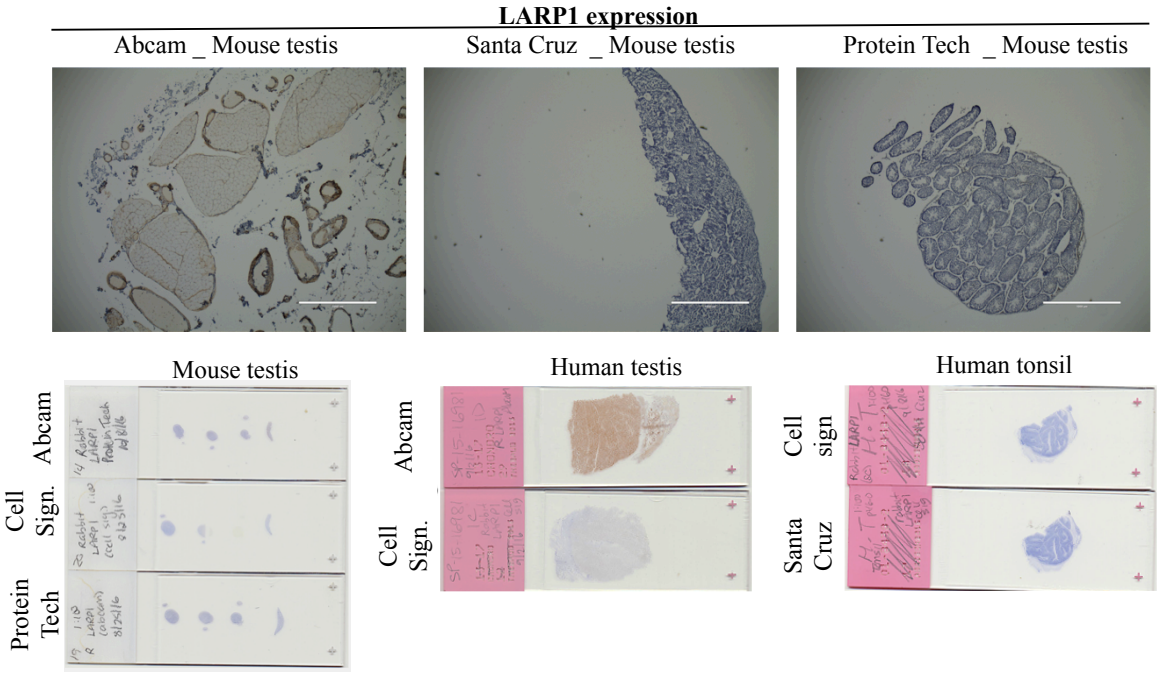

Figure 25. Representative images of LARP1 staining by immunohistochemistry using commercially available antibodies. Weak or non-specific staining observed in different mouse tissues and relatively strong non-specific binding for LARP1 antibody from Abcam in mouse and human testis (**Up**). As seen in overall view of all slides stained (**Bottom**), some LARP1 antibodies tested (Cell Signaling, Abcam, Protein Tech) did not specifically detect LARP1 in mouse tissues, while others (Santa Cruz and Cell Signaling) did not detect LARP1 even in human tonsils.

Effects of LARP1 knockdown on cell proliferation and migration

Cell proliferation

OVCAR8 cells were transduced with shRNA lentivirus silencing LARP1 (shLARP1-2 and shLARP1-4), or a negative control lentivirus (shSCR); LARP1 silencing was confirmed by Western blotting. Using CCK-8 assays, the results showed that LARP1 silencing significantly attenuates the proliferation of OVCAR8 cells. To further confirm this observation, cells were periodically counted using a hemocytometer. The results demonstrate that LARP1 silencing decreases OVCAR8 cell ability to proliferate.

It was interesting to observe that the culture medium (DMEM 10%FBS with 1%P/S) was remarkably changing color with time for the control cells (shSCR) while remaining pink for knockdown cells (shLARP1-2 and shLARP1-4). This experiment was repeated in 6 well plate where cells were covered with 2mL medium and the results show similar effect. The change in color from pink to orange/yellow suggests a pH changed caused by metabolites released by growing (proliferating) cells. These results indicated that LARP1 silencing decreases cell proliferation for OVCAR8 cells (**Figure 26**).

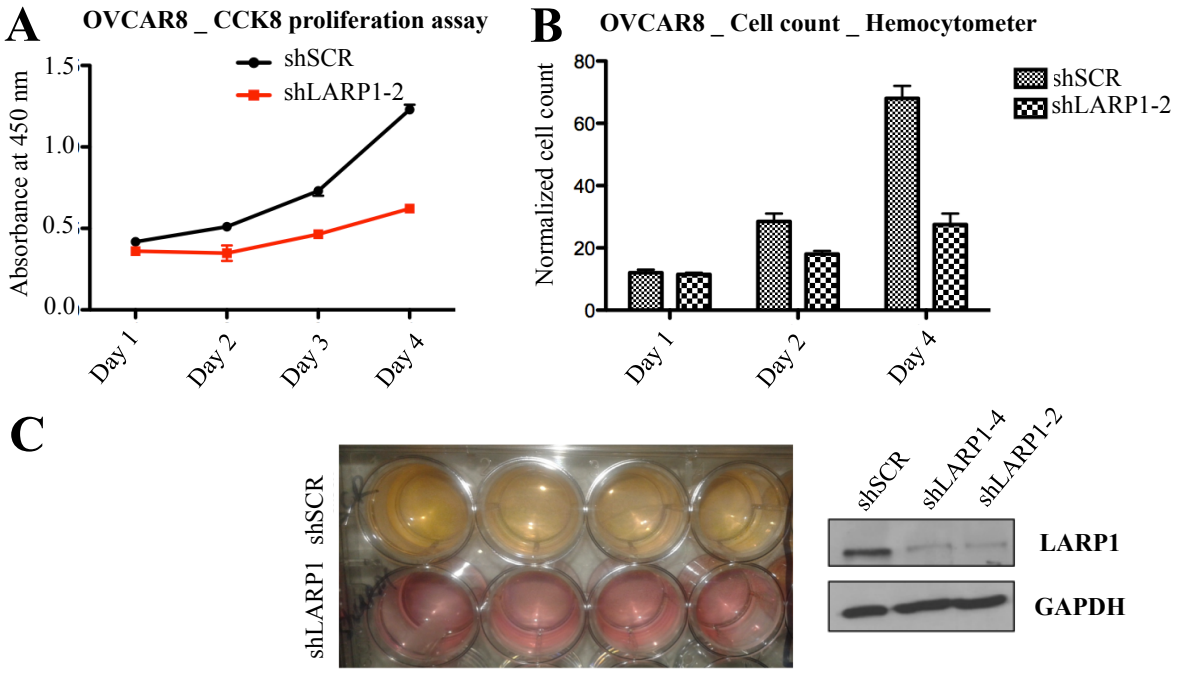

Figure 26. LARP1 depletion decreases cell proliferation in OVCAR8 cells. Effect of LARP1 silencing on cell proliferation was assessed by Cell Counting Kit-8 assays (**A**) and cell counting using hemocytometer (**B**). Culture medium color change was monitored periodically and LARP1 silencing was confirmed by Western blotting (**C**). Error bars represent standard error of the mean.

Cell migration

Given that LARP1 is a novel target of mTOR and mTOR is known to be an effector of cell migration and invasion, I decided to test the effect of LARP1 knockdown on OVCAR8 cell migration. I compared the migration ability of scrambled (shSCR) and LARP1 knockdown cells (shLARP1) using the scratch assay. The results show that LARP1 depletion significantly reduced cell migration of OVCAR8 cells (**figure 27**).

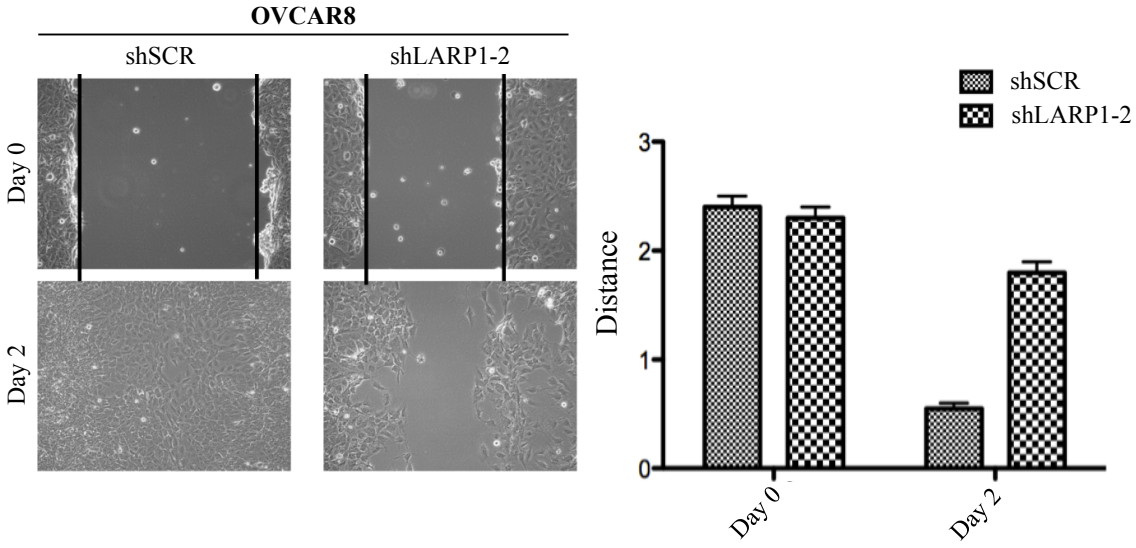

Figure 27. Effect of LARP1 knockdown on OVCAR8 cell migration. Phase contrast micrographs (**left**) showing OVCAR8 cells immediately (0 h) after scratch and two days (48 h) after scratch. Black lines indicate wound edges. Quantified and normalized migration distances are compared per time point (**right**). Error bars represent standard error of the mean.

Effects of LARP1 silencing on oncolytic viral infection

The Vesicular Stomatitis Virus (VSV) is one the leading oncolytic viruses undergoing clinical trials. It would be interesting to determine the feasibility of a combined therapeutic strategy using oncolytic viruses and drugs such as mTOR inhihbitors. As a novel target of mTOR, LARP1 might play a significant role in such approach. To assess the effect of LARP1 silencing on oncolytic viral infection, scrambled OVCAR8 cells (shSCR) and LARP1-depleted cells (shLARP1) were infected VSV Δ51 containing GFP. LARP1 silencing was confirmed by Western blotting. The results show increased viral infection in LARP1-depleted cells (**Figure 28**).

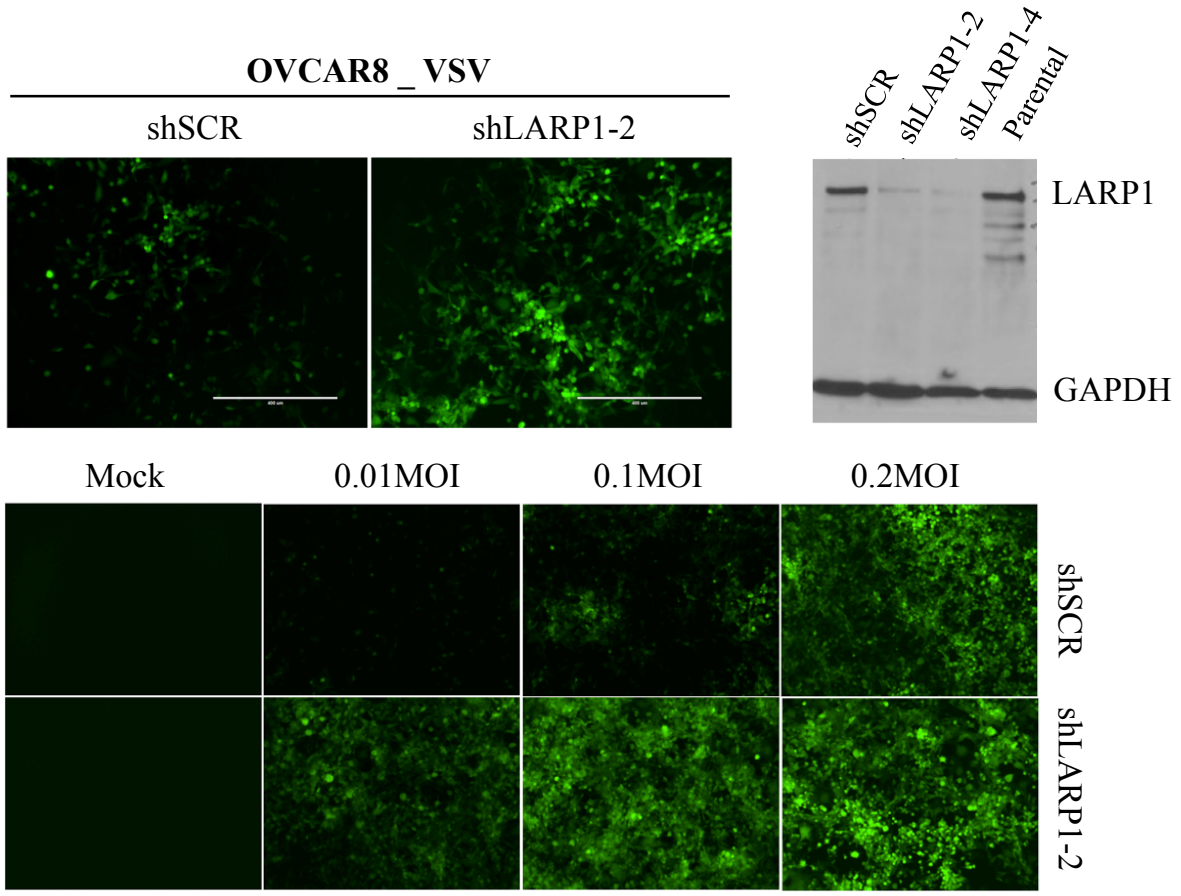

Figure 28. Enhanced VSV replication in LARP1 knockdown OVCAR8 cells. (**A**) OVCAR8 stably knockdown (shLARP1) and sh Scrambled OVCAR8 cells (0,4 × 10^6cells/well in 12-well plate) were infected with VSV Δ51-GFP at the indicated volume. Forty-eight hours post-infection, the GFP signals were photographed under fluorescent microscope. (**B**) Cell lysates from similarly OVCAR8 cells were analyzed by Western blotting for LARP1 and GAPDH to confirm LARP1 depletion. Indicated titers were deduced from volumes of virus used for infection.

Role of LARP1 in drug response

As a downstream target of mTOR, it is plausible that LARP1 plays a role in the response of cancer cells to different therapeutic strategies, especially those based on mTOR inhibition. Therefore, LARP1-depleted OVCAR8 cells (shLARP1) and their scrambled counterparts (shSCR) were treated with Rapamycin and Torin1, two well characterized mTOR inhibitors. Interestingly, increased apoptosis was observed in LARP1-depleted cells compared to scrambled cells. The results demonstrate that LARP1 silencing renders OVCAR8 cells more sensitive to the drugs as seen in **Figure 29.**

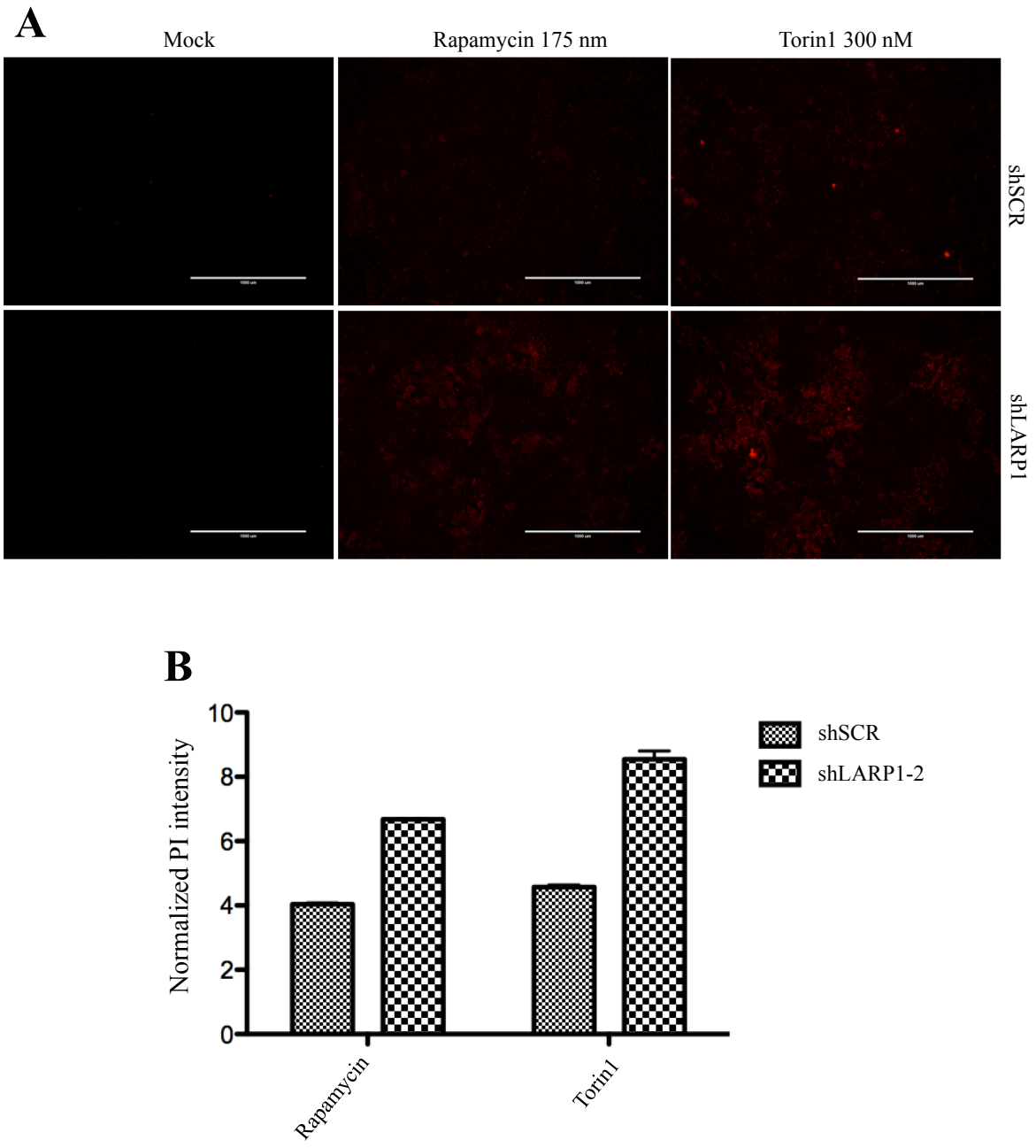

Figure 29. Effect of LARP1 silencing on response to mTOR inhibitors. Scrambled OVCAR8 cells (shSCR) and LARP1-depleted OVCAR8 cells were treated with 175 nM Rapamycin or 300 nM Torin1. Propidium Iodide (PI) (red color) was used to stain for dead cells by immunofluorescence. The intensity of PI was quantified using Image J. Error bars represent standard error of the mean.

4. DISCUSSION

LARP1 EXPRESSION AND CANCER PATIENT SURVIVAL (Kmplot)

Using the KM plotter for survival analysis, I provide evidence for a significant correlation between high LARP1 mRNA expression in gastric cancer patients and visibly improved overall survival outcomes (**Figure 6**). Interestingly, these findings corroborate with LARP1 protein expression in gastric cancers where tissue microarray pictures demonstrate a higher level of LARP1 in normal tissues compared to gastric cancer patients (**Figure 17**). Notably, a study by Cheng and colleagues has previously suggested that LARP7 also is highly expressed in normal stomach tissues but lost in stomach cancer tissues [72, 73]. Moreover, survival data from the Kaplan-Meier plotter (www.kmplot.com) indicates that gastric cancer patients survive much better with higher vs. lower LARP1 expression level. These findings support the conclusion that LARP1 and possibly LARP7 hold a potential of acting as tumor suppressors for gastric cancer.

I was very much interested in reproducing the results from Hopkins and colleagues [67] suggesting that high LARP1 mRNA expression correlates with poor survival of ovarian cancer patients. The authors provided results from the Kaplan-Meier plotter (www.kmplot.com) to support their claim. Surprisingly, the results of my analysis (**Figure 7**) revealed that there is no significant correlation between high expression of LARP1 and ovarian cancer patient survival in all the versions of the Kaplan-Meier plotter available at the time of the analysis (version 2011, version 2013 and version 2015). Moreover, the exact same query inputs were used by Hopkins and colleagues (confirmed by the number of patient). Thus, my conclusion is that the survival data presented in the supplementary figures by Hopkins and colleagues are not repeatable as described (**Figure 7**).

Surprisingly, when comparing LARP1 mRNA expression with the expression of other genes

such as the components of the mTOR pathway or other well-known altered genes in specific cancer types, the results show that even high or low mRNA expression of genes known as tumor suppressors or oncogenes in certain cancers does not necessarily correlate with better or poor prognosis respectively (**Appendices _ Kmplot**). Thus, I consider that the survival rate alone cannot, especially from in-silico experiments, be used to predict the role of a given gene in cancer progression (as tumor suppressor, oncogenic or altered).

LARP1 mRNA expression in cancer

Consistent with the idea that mRNA expression of potential tumor suppressors or proto-oncogenes should be reduced or elevated (upon activating mutations and vice versa) respectively in cancer tissues when compare to their adjacent normal tissues, I aimed to compare the expression level of LARP1 in different cancer cell lines using the Cancer Cell Line Encyclopedia (CCLE). As expected, the results show that prostate cancer cell lines have the lowest expression level of LARP1 compared to most tumor cell lines. Consistent with the Human Protein Atlas results (**Figures 8-9 and Figure 16**), it clearly appears that LARP1 is lost in prostate cancer at the mRNA and at the protein level. The significant variation in LARP1 mRNA expression level in various cancer cell lines indicates that LARP1 is a potential therapeutic target for various cancers (**Appendices _ LARP1 mRNA expression**).

To further characterize the mRNA expression of LARP1 in normal and adjacent tumor tissues, I interrogated the Oncomine database. No relevant difference in LARP1 expression was noticed between normal and cancer tissues (Fold changes < 1.5 as seen in **Figure 10**). The main challenges when extracting data from the Oncomine database was to find statistically significant differences or correlations and assembling comparable data from the same study. These two constraints do not only reduce the number of patients included in the analysis, but also reduces the chance to retrieve significant difference in gene expression.

LARP1 MUTATIONS AND AMPLIFICATION IN CANCER

During analysis of LARP1 mRNA expression using different databases, it became clear that mRNA expression does not always correlate with that of protein. I then aimed at exploring the gap between gene/mRNA expression and functional protein expression. Post-transcriptional and post-translational modifications are known to play a key role in the functions of mRNA and protein, respectively. As cancer cells rely on the ability to truncate gene expression and cell growth or to favor their aberrant proliferation, a gene expression event such as gene amplification is an ultimate upstream precursor of sustained tumorigenesis. Szpankowski and colleagues [74] conducted experiments to assess amplification patterns of various human cancers in order to establish a classification of cancers based on amplification mechanism. As previously stated by Myllykangas and colleagues [75], mutations that were shown to "increase the copy number of a specific DNA segment", named DNA amplifications, are often observed in human cancers. Thus, the overproduction and overexpression can be explained by the fact that the genes are transcribed on every copy and translated. This process does not often happen in normal cells, but its occurrence has been often observed in cancer cells. Thus, if the amplified region contains an oncogene or a tumor suppressor, the resulting overexpression of the specific gene would most likely lead to uncontrolled cell growth which is a hallmark of cancer cells [15,75]. Many clinical treatments have been and are being designed to specifically target cells which overexpress the protein product of genes such as *MYC* or the *HER2* oncogenes, which are highly amplified in breast and ovarian cancers [76, 77].

Moreover, Bredel et al. [78] have identified gene amplification among the main reasons for cancer drug resistance. A well-known example of this resistance mechanism is the so-called *MDR1* (Multi drug resistance 1) gene whose protein product acts as an ATP-dependent efflux pump at the cell membrane; actively transporting molecules including cancer drugs into the

extracellular milieu. This ejection renders the drug practically ineffective [79]. Because mTOR expression is often deregulated in cancer and many cancer therapeutic strategies aim to target the mTOR pathway have shown various limits, I projected that as a key target of mTORC1, LARP1 (and it genetic alterations) could play a significant role in potential combined therapeutic strategies based on the mTOR pathway. Thus, I analyzed genetic alterations which can occur with LARP1 in normal and cancer cells; including mutations, copy number variation and amplification. I extended my assessment to some other specifically selected genes for comparison. Globally, the results demonstrate that LARP1 gene expression is not significantly altered or amplified in most cancers (**Figure 11**). Coincidentally, most research papers published previously have not mentioned any relevant mutation or any other alteration with LARP1 gene expression, which could explain its potential role in cancer progression. It would be interesting to determine if other modifications such as methylation could explain the role played by LARP1 in the progression of prostate cancer for example. Surprisingly enough, LARP1 expression was found to be amplified as much as PTEN (31%) in the neuroendocrine prostate cancer study conducted by Cornel and colleagues (**Figure 12** and **Figure 13**).

Neuroendocrine prostate cancer (NEPC) encompasses different clinical aspects from the de novo initiation of small cell prostatic carcinoma to a fully transformed phenotype known to often arise from typical prostate adenocarcinoma tissues. It has been proposed that resistance to the signaling inhibition of potent androgen receptor is associated with the emergence of aggressive form of castration-resistant neuroendocrine prostate cancer (NEPC).

The main characteristics of NEPC tumors are the loss of AR expression, loss of RB expression or copy number, amplification of N-myc and more interestingly the activation of the phosphoinositide 3-kinase (PI3K) pathway [80]. Again, it is not very clear why LARP1 may be specifically amplified in this prostate cancer subtype. Nevertheless, it is noteworthy in

this context to remember that LARP1 lies downstream the PI3k/Akt/mTOR pathway. This pathway endorses many cancer-related interactions including inhibition by the tumor suppressor PTEN. This could partially explain why LARP1 is amplified in NEPC cases.

I suggest that a more intensive characterization of physico-chemical properties of LARP1 and its molecular functions and interactions (for example by mass spectrometry and immunoprecipitation), at this step, would help to elucidate which one of its features play an important role in cancer progression. It's not excluded that posttranslational modifications such as phosphorylation or other genetic alteration might eventually lie behind the role of LARP1 in cancer progression and in TOP mRNA translation.

LARP1 NETWORK ANALYSIS

Although gene expression profiling provides tremendous number of important data on cancer mechanism, assigning a biological significance and mapping networks of all newly found interacting genes remain a challenge. Based on the assumption that tumor cells use preexisting cell pathways by modifying them and not by creating new ones, many bioanalytical and bioinformatics tools have been developed to predict molecular networks. The STRING database was used to predict the interactions between LARP1 and other genes [81]. The network analysis show that LARP1 potentially interacts with several ribosomal proteins downstream the mTOR pathway, as previously reported [67].

Interestingly, I also observed that LARP1 is predicted to interact with some microRNAs such as Lin28A (**Figure 14**). The latter observation substantiates recent findings [71] that emphasized the interaction of microRNAs and LARP1 as crucial element for the control of cell proliferation, cell migration and cell invasion in some cancers. More than 50 micro-RNAs have been identified as playing a role in prostate cancer. Among those microRNAs, some have been shown to interact with LARP1 and supposedly conferring a proto-oncogene role to

LARP1 in prostate cancer [82].

There is a sustained evidence that LARP1 acts downstream of the mTOR pathway to control TOP mRNA translation, but it also appears that LARP1 plays certain mTOR-independent roles in cancer by interacting with different genes including miRNAs and other RBP-coding genes. Nonetheless, one should not underestimate potential pitfall of gene-by-gene interactions predicted by in-silico modeling approach as they are mainly founded on hypotheses-based predetermined data. These predictions do not necessarily prove that such interactions are possible or feasible in human tissues. Moreover, molecular functions and interactions can vary depending on the cell or tissue, which considerably limits the scope of conclusions to be made from database network analysis. Functional testing and integrated studies (regulator and metabolic) are critically needed to confirm these predicted interactions and provide additional insights on how other proteins interact with LARP1 and vice versa.

LARP1 EXPRESSION IN THE HUMAN PROTEIN ATLAS

The initial focus of this study was to compare the expression of LARP1 in normal versus adjacent cancer tissues, a prerequisite for a potential biomarker prognostic with possible diagnostic value. To do so, the Human Protein Atlas was queried for LARP1 expression level in different cancer and normal tissues. More than 4000 pictures of tissue microarray pictures for 6 cancers and nearly 15 proteins were assembled and classified by age, grade and sex of patients when available.

Immunohistochemistry scoring of different normal and cancer tissues stained for LARP1 were re-evaluated with two independent pathologists and confronted results with related literature when available. Overall, I found that LARP1 is highly expressed in normal prostate and normal stomach tissues while no significant change in expression was observed for other cancers (**Figure 15**). LARP1 expression is elevated in the glandular cells of prostate tissue

(**Figure 16**), which strongly emphasizes its role as potential tumor suppressor and translation repressor as suggested by Fonseca and colleagues [26]. However, I noticed that the staining of LARP1 antibodies used for immunohistochemical experiments are far from being optimal; especially the staining quality of some LARP1 antibodies used in the Human Protein Atlas (**Appendices _ Human Protein Atlas**). This demonstrates, once again, that results from database are only preliminary and the methods used might have some significant limitations which require a critical assessment before making strong conclusions from resulting findings.

The protocols used for immunohistochemistry as described in the Human Protein Atlas website (www.proteinatlas.org) result in a black-brown staining, localized where an antibody binds to its corresponding antigen. Stained sections are furthermore counterstained with hematoxylin to allow visualization of tissue microscopical features (blue coloring). This image-based study has shed light on the expression pattern of LARP1 in different cancers. However, some limitations are to notice. First, the ideal comparison would include normal and adjacent cancer tissues and tissue subtypes from the same patient. The comparison of expression level is made between normal tissues and adjacent cancer tissues obtained from different patients. The rationale of this comparison was to assess the expression of LARP1 in specific subcellular part such as glandular cells for prostate tissue where more than 90 % of prostate adenocarcinoma are expected to be initiated [83].

Normal tissue is a complex structure composed of various cell subtypes, most of which may or not represent the actual cells of cancer origin. However, comparing tissues from different patients provided valuable information on where LARP1 is overexpressed or lost. Another potential shortcoming of our systematic protein expression study was the validation of the LARP1 antibodies used for the immunohistochemical staining in the Human Protein Atlas project. Although the Human Protein Atlas holds a six-step quality assurance mechanisms for antibody validation strategy, it was surprising to discover that two of the three LARP1

antibodies (HPA051819 and HPA051397) used in the Human Protein Atlas project were not validated by Western blotting. Moreover, the third one (CAB015222) has been discontinued (www.proteinatlas.org). For Western blotting results used in the Human Protein Atlas, only bands corresponding to the predicted size (with or without additional bands) are supported and presented if the precision rate is within a range or +/-20%. Western blotting images of antibodies showing uncertain data are not shown; "uncertain" including all cases not corresponding to the two supported cases mentioned above. One can suppose that the results of Western blotting validation performed by the Human Protein Atlas Project Quality assurance system did not show any correct size within the expected range for the two "uncertain "LARP1 antibodies (HPA054819 and HPA051397). By contrast, my results (see **antibody Validation section**) demonstrated that the LARP1 antibody HPA051397 does not only specifically stain for LARP1 in HEK293T and OVCAR8 cells by Western blotting, but also by Immunofluorescence. Fortunately, feedback from the research community is highly appreciated by the Human Protein Atlas in order to keep continuous curation of protein and antibodies data. My LARP1 antibody validation results will be provided to the Human Protein Atlas, as agreed, to help update the antibody validation data for LARP1 protein currently available at the HPA website (www.proteinatlas.org).

LENTIVIRAL TRANSDUCTION

To determine the effect of lentivirus-shLARP1 and shSCR on the expression of OVCAR8, HeLa and HEK293T cells, protein levels were analyzed by Western blotting. 48h and 72h after transfection, filtered medium containing lentivirus (LV-shLARP1 and LV-shSCR) were used to transduce OVCAR8, HEK293T cells or HeLa cells. Stably transduced cells were selected on puromycin for 5 days, non-transduced cells were killed (**Figure 19**). Puromycin concentration was chosen based on standard curves determined by literature review. Most

published results indicate that 5ug/mL of puromycin was used to select HEK293T and HeLa cells [84].

After puromycin selection, cell pellet was collected and LARP1 silencing was confirmed by Western blotting as shown in **Figure 21**.

Notably, I noticed that all knockdown cells (shLARP1) from different cell lines were growing much slower than parental and scrambled control cells. This observation was even more visible in OVCAR8 cells. Consistent with the suspected role of LARP1 and mTOR in cell proliferation ability, I first seeded equal number of cells for both scrambled and knockdown cells, and then repeated the experiments seeding at lower density for scrambled cells to obtain equal confluency at growing phase. The last experiment allowed me to perform functional assays at the same confluency on the one hand, and confirm the role of LARP1 in cell growth and proliferation on the other hand.

Cells with depleted LARP1 (shLARP1) grew and proliferated significantly slower than parental cells, as confirmed by cell counting and color medium change results (**Figure 26**). I verified the knockdown effect of LARP1 in OVCAR8 cells; I found that after 3 passages, LARP1 expression did not come back on LARP1-depleted cells (shLARP1). I predict that *in-vivo* knock-out experiments, such as CRISPR cas9 for LARP1, would yield information on the role of LARP1 on cell growth in that cells lacking LARP1 may perhaps not grow at all. By contrast, if such cells (LARP1 knockout) grow normally, it's more likely that the slow proliferation observed is not permanent and could be jeopardized by compensatory cellular effects. However, given the complexity of cell functions and a plethora of mechanism involved, it's not surprising that even LARP1 Knockout cells or long term (more than 10 passages) LARP1 knockdown cells would find alternative and compensatory mechanisms to allow normal proliferation despite the absence of LARP1. My experiments culturing LARP1-

depleted cells show that LARP1 plays a role in cell proliferation. However, this conclusion would make more sense if LARP1 is knocked down in different cell lines and cell proliferation compared between scrambled control cells and LARP1-depleted cells.

LARP1 ANTIBODY VALIDATION

When defining the main objectives of this study, one of the main goals was to perform immunoassays in order to characterize the expression of LARP1 in normal versus cancer tissues. However, finding the right antibody to use for these assays soon became a considerable challenge. This led me to a profound rethinking of the choice and the role of antibodies to be used for immunoassays.

Beneath the complexity on the choice of antibodies lie a limited number of well validated antibodies and a defined standard to validate specificity of antibodies. A previous effort by Bourbeillon and colleagues [85] resulted in a proposal to formalize report standard on affinity binder reagents including antibodies. However, this proposal, named MIAPAR (minimum information about a protein affinity reagent) does not provide detailed approaches for specific applications [85]. Each protein has a specific conformation and offers variable target accessibility. Thus, antibodies targeting such proteins may perform well in one application but might bind inadequately in a different application. Moreover, the level of off-target and non-specific binding is considerably affected by the ratio of the target protein within the whole sample to other proteins. Indeed, good staining in Western blotting does not always guarantee a good performance of the antibody when used for immunofluorescence, immunohistochemistry or sandwich ELISA. Thus, approaches for antibody validation should be carried out with consideration of the experiment context and for each application specifically.

The fact that LARP1 antibodies used in the Human Protein Atlas are not validated by Western blotting perfectly exemplifies the issue related to antibody validation mentioned above. Moreover, the third LARP1 antibody used in the Human Protein Atlas project and in some publications (CAB015222) has been simply discontinued; rendering impossible any tentative to reproduce the results from the Human Protein Atlas (www.proteinatlas.org).

With consideration of all cited above, I decided to validate selected commercially available LARP1 antibodies including those used in the Human Protein Atlas and validate them by Western blotting, immunofluorescence and eventually by immunohistochemistry. The results demonstrated that some LARP1 antibodies (Abcam, Sigma and Cell Signaling) specifically detect the protein while others (Santa Cruz) show some level of non-specific binding or very weak bands when used for Western blotting. As seen in **Figure 21**, it is evident that these antibodies specifically recognize LARP1 protein in human cell lines but poorly work in mouse tissues (**Figure 22**). This once again demonstrates that validation must be done for each specific species or sometimes even for every single experiment design.

In addition, I decided to validate those tested antibodies by immunofluorescence. Although Western blotting is the most used assay to confirm antibody specificity (www.antiboypedia.com), the rationale behind this choice was the consideration of the structural conformation of proteins. It's worthy reminding that proteins are usually partly or wholly denatured during immunoassays. The structure of the epitope (specific peptide sequence on the antigen) which is recognized by the paratope (peptide sequence within the antibody, which recognizes the antigen) can be linear (located on an available surface and presented as a set continuously aligned amino acids) or conformational (non-linear alignment and presented at three-dimensional structure). Western blotting strategies rely on the fact that the majority of epitopes can still be recognized after protein denaturation by SDS. However, this linearized structure does not always correspond to the real and active protein

conformation. It's well known that the majority of antibody-antigen interactions strongly rely on the conformational structure of the antigen [86]. Thus, using immunofluorescence or immunohistochemistry became a necessity to validate the selected antibodies as the protein is nearly kept at it conformational structure during such assay.

My results (**Figure 23**) show that LARP1 antibodies from Abcam and from Sigma specifically recognize LARP1 in HEK293T and HeLa cells by immunofluorescence. LARP1 antibody manufactured by Santa-Cruz shows a poor specificity using immunofluorescence. Although LARP1 antibody from Cell Signaling specifically detected the protein by Western blotting, the staining was less specific by immunofluorescence compared to the ones from Abcam and from Sigma.

Using immunohistochemistry, OVCAR8 cell pellets were fixed on slide using formalin and stained for LARP1 expression comparing scrambled (shSCR) and knockdown cells (shLARP1). Unfortunately, my results indicate poor detection and considerably high signal to noise ratio (**Figure 24**). Nevertheless, my impression is that the most promising staining could be obtained with LARP1 antibodies from Sigma and Abcam. This is based on the results from Western blotting and immunofluorescence. However, the cell pellet strategy is not the optimal assay for antibody validation as the design of experiment must be fastidiously optimized at each step (blocking, antibody concentration, antigen retrieval method, counterstaining and microscopy) to avoid nonspecific binding caused by the smear of the nucleus as seen in the negative controls (Hematoxylin counterstaining in OVCAR8 cell pellet) (**Figure 24**). Another limitation of the cell pellet strategy is the potential heterogeneity of mixed cells within the pellet, which could lead to irregular staining and false positive or false negative results due to a non-homogenous surface of cells. An alternative approach would be to fix cells on culture plate using PFA and obtain a homogenous and regular cell surface as done for immunofluorescence and then perform immunohistochemistry. However, not all cell lines

fixed with PFA would remain attached after heat treatment for antigen retrieval (temperature > 95° C).

Given the predominant role of antibodies in immunoassays, our validation approach shed light on the tremendous importance of assessing reagents and optimizing design of experiments accordingly to ensure experiment robustness and repeatability. Should Western blotting fail to validate antibodies, one could and should use other immunoassays to circumvent and confirm results before concluding on the antibody functionality and vice versa.

Effects of LARP1 knockdown on cancer cells

Cell proliferation and cell migration

The previous results suggest that LARP1 plays a role in cell growth and cell proliferation in cancer cell lines such as OVCAR8 [71]. It has been previously suggested that LARP1 plays an important role in the progression of prostate cancer, non-small lung cancer, HCC and cervical cancer [87-88]. Cell proliferation together with increased invasiveness can be considered as the two most prominent of all hallmarks of cancer cells corresponding to the majority of cancer-related deaths [15]. To provide more evidence of the hypothesis that LARP1 plays a role in cell proliferation and cancer progression, specific functional experiments were carried out using OVCAR8 cells. First, the difference in cell proliferation between LARP1-depleted cells and scrambled cells was assessed. Afterward, migration and proliferation assay were used to compare between scrambled and LARP1-depleted cells. The results showed that LARP1 silencing in OVCAR8 cells considerably decreased cell proliferation and migration ability (**Figure 26**). Notably, the results of cell counting with hemocytometer and CCK-8 proliferation assay demonstrated that LARP1-silenced cells proliferate less than scrambled cells. Much is known about the role of molecular targets as key actors in strategies to diagnose and treat cancer. The coherence of these results gives

confidence that LARP1 can be a desirable therapeutic target in cancer treatment. The discovery of the role played by mTOR within the phosphatidylinositol-3-kinase (PI3K/AKT/mTOR) pathway received widespread attention and has since driven many groups to deeply assess how this pathway controls protein synthesis and mRNA translation, affecting both cell growth and proliferation [89-90].

Predictions that LARP1 depletion affects cell growth and proliferation were previously demonstrated by Ye and colleagues using colony formation assays [91]. However, in their studies, Ye and colleagues did not clearly establish an interaction between mTOR and LARP1 predicted to happen through RAPTOR. It's not totally sure whether LARP1 unequivocally mediates cellular growth and proliferation control through the mTOR pathway. In this context, it is noteworthy that previous studies have demonstrated a positive correlation between LARP1 and the proliferating cell nuclear antigen (PCNA) in colorectal cancer. Immunohistochemistry and Western blot analysis of LARP1-silenced cells indicated that LARP1 absence directly reduces PCNA expression in colorectal cancer [91]. Even more, LARP1 mRNA level has been reported to be much more abundant in colorectal cancer cells than in adjacent normal cells [91]. The proliferating cell nuclear antigen is often used as a marker of cell proliferation. This important accessory protein of DNA polymerase plays an important role in DNA replication as well. Assessing the effect of LARP1 silencing on PCNA expression on cancer cell lines is definitely an approach to consider. However, based in my experiments, I anticipate that LARP1-depleted cells would probably proliferate much less than scrambled cells. This unequal proliferation rate may render comparisons very difficult as cells will reach desired confluency for the study at different times.

Notably, Hopkins and colleagues [67] have noticed a significant alteration of functions linked to cancer in LARP1 knockdown transcripts. The authors claimed that LARP1 knockdown directly affected cell growth, cell proliferation, cell death and cell survival. Most strikingly,

LARP1 silencing was associated by the authors with alteration of apoptosis-related genes. Pro-survival genes such as *BCL2*, *ERBB3* and *AKT3* were shown to be negatively regulated by LARP1 while pro-apoptotic genes such as *BIK*, *TNF* and *DAPK2* were positively regulated by LARP1 in epithelial ovarian cancer [67]. Thorough assessment of the effect of LARP1 silencing on cancer cells, it appears that LARP1 functions include the control of cell proliferation, cell migration and cell invasion in different cancers.

However, depending on different parameters and conditions such as the type of cancer involved or the phosphorylation status, LARP1 may positively or negatively regulate the cellular functions cited above. Altogether, it seems very likely that LARP1 has the potential to be used as biomarker and facilitates the development of new cancer therapeutic strategies; but its mechanism of action must be elucidated before then.

Effect of LARP1 depletion on oncolytic viral infection

Since the first documentation of cancer in Egyptian papyrus by Imhotep in 1550 BC, tremendous effort has been deployed toward cancer eradication. However, given the complexity of the disease, considered by many as a set of diseases rather than one disease itself, it's likely that the way to end cancer is not yet clearly made.[3] Different strategies have been developed to fight cancer progression among which surgery, radiation and chemotherapy. However, these strategies have many limitations among which tumor resistance. Because tumor cells are able to evade T-cell recognition and secrete immunosuppressive cytokines, a newer tendency aimed to include the immune system in the fight against cancer.[4]

[3] www.cancer.org
[4] www.dendron.com

The discovery and development of oncolytic viruses as biological tools providing tumor-specific cell lysis and immune stimulation has attracted widespread attention of different research groups toward cancer immunotherapy. The effectiveness of oncolytic viruses has been and is being assessed through several ongoing clinical trials worldwide. Thus, immunotherapy involving oncolytic viruses is currently gaining interest not only in the scientific community but in clinical spheres as well.

Notably, it has been well recognized that the combination of different cancer therapy strategies is a highly promising approach in the fight against cancer; given the complexity and the heterogeneity of tumor cells. Treatment strategies using mTOR inhibitors such as rapamycin have shown several limitations among which drug resistance and partial inhibition. Although better inhibitors of the mTOR pathway have been developed, drug resistance still remains an issue for cancer patients treated with mTOR inhibitors. Therefore, I suggest that LARP1 as a key player downstream the mTOR pathway could be relevant in a combined therapeutic strategy using mTOR inhibitors and oncolytic viruses; eventually leading to enhanced drug effectiveness. Perhaps LARP1 can be a determinant element in drug response due to its role in TOP mRNA translation. Thus, I aimed at testing the effect of LARP1 depletion on the viral infection ability of different oncolytic viruses available such as the Herpes Simplex Virus (HSV), Toscana Virus, Punta Toro Virus and the Vesicular Stomatitis Virus (VSV). After a preliminary selection and depending on the availability of the viruses, the effect of LARP1 silencing was tested on VSV infection only. Surprisingly, the results show that VSV infection is greatly enhanced in LARP1-silenced OVCAR8 cells (**Figure 28**). To my knowledge, this is the first study assessing the effect of LARP1 silencing on oncolytic viral infection using VSV. Vesicular stomatitis virus belongs to the Rhabdoviridae family and has been characterized by a plethora of studies [92-93]. Five genes encode the 11kb genome of VSV. The virion is enveloped and encodes for 5 proteins which are the nucleocaspid

protein (N), the matric protein (M), the phosphoprotein (P), the G protein and the large polymerase (L). Interestingly, VSV is known to replicate within the cytoplasm where LARP1 is also found in most cells. VSV is also known to grow fast and to reach considerably high titers in infected cells, rendering it purification easier as large amount of viral proteins are present in cell lysates [94-95]. Why exactly does VSV infection increase in LARP1-silenced OVCAR8 cells? Any answer to this question would surely include the mechanisms of VSV infection on the one hand and the mechanism of LARP1 function on the other hand.

Considering the discrepancy going on about the exact role of LARP1 (repressor or activator) in mRNA translation and the non-elucidated interaction pattern between LARP1 and mRNA transcripts (3'UTR and 5'UTR), aiming to answer this question would require a full complete study. Moreover, despite the fact that VSV is relatively simple, easy to manipulate and has been assessed in many cases including melanoma, glioblastoma, breast cancer and others [96], a considerable number of scientists strongly argue on it oncoselectivity [97].

Hopkins and colleagues [67] have previously suspected the role of LARP1 in chemoresistance for ovarian cancer cells where LARP1 transient inhibition led to reduced resistance and increased apoptosis. However, no association was clearly established by the authors between increased apoptosis due to LARP1 silencing and changes in cell cycle. The results emphasized the idea that LARP1 silencing leads to enhanced apoptosis as shown in OVCAR8 cells (**Figure 29**) using Propidium Iodide (PI) - a red marker used to identify dead cells by intercalation between DNA bases. I suggest that more studies combining different oncolytic viruses and different mTOR inhibitors or LARP1 silencing in cancer cells would provide more evidence on the potential tumor-suppressor or proto-oncogenic role of LARP1.

Nevertheless, my bioinformatics findings on LARP1 expression together with results demonstrating the effect of LARP1 knockdown on different cellular functions unequivocally

convince me about the potential role of LARP1 as a relevant target for cancer treatment using different therapeutic strategies.

5. CONCLUSIONS AND PERSPECTIVES

In the past decades, understanding the mechanism of cancer progression and establishing cancer therapeutic strategies have undergone an extraordinary expansion, leading to the discovery of several chemotherapeutic and immunotherapeutic strategies. However, early diagnosis remains a significant issue in order to reduce cancer-related mortality and the number of metastatic patients. The diagnosis of prostate cancer for example is often based on biomarkers such as PSA which provides low specificity leading to a significant number of unnecessary biopsies and mistaken diagnoses jeopardizing the welfare of clinical management as well as the trust of cancer patients.

Given the heterogeneity of tumor cells and the complexity of mechanisms involved in cancer, there is a need for refined treatment and diagnosis strategies incorporating various biochemical aspects to yield a better efficacy in cancer treatment on the one hand and strengthen the effort toward biomarker discovery effort on the other hand. While the design of experiments remains critical for such effort, the choice of reagents such as validated antibodies must be subject to a higher degree of scrutiny to ensure repeatability of the experiments.

It is important to consider that LARP1 stands downstream the mTOR pathway, which is also often altered in almost all cancers and was previously suggested to control the translation of ribosomal proteins. Moreover, LARP1 expression has been found to be altered in various cancer tissues compared to their normal adjacent tissues. As such, LARP1 is a potential bridge explaining how mTOR controls the translation of TOP mRNAs leading to cell proliferation. Based on in-silico databases, LARP1 is differently altered in different cancers at

the mRNA and at the protein level. While being consistently lost in prostate cancer tissues, LARP1 seems to be overexpressed in many other cancers.

Intriguingly, it's still not clear enough how exactly LARP1 binds to mRNA transcripts, and what determines its putative dual role as repressor and activator of TOP mRNA translation. This discrepancy has led to several contradicting published results where different authors used different tools to elucidate the same mechanism. As to add more difficulties to this existing discrepancy, I noticed that the analysis of in-silico databases and the choice of reagents such as antibodies used in many publications to characterize LARP1 protein are not always subject to a higher degree of scrutiny, leading to unrepeatable results.

Nevertheless, I believe that LARP1 plays a significant role in protein synthesis, both by activating and repressing TOP mRNA translation. I suggest that this double role depends on many factors including but not limited to the phosphorylation status of LARP1, mTOR-independent interactions of LARP1 with other RBPs or microRNAs and perhaps tissue-specific or compassing cellular mechanisms regulating LARP1 expression in tumor cells.

The results of LARP1 antibody validation and LARP1 silencing experiments should inform the ongoing debate by the LARP1 scientific community on which antibodies are to be used and the expression level of LARP1 in different cancers. A deeper characterization of the DM15 motif and further in-vivo experiments with LARP1 knock-out with CRISPR method for example, together with assessment of drug response will surely provide new insights on potential oncogene-related or tumor suppressor roles of LARP1 as therapeutic target. Such study will also help elucidate the exact mechanism of LARP1 interaction with the mTOR pathway and with other non mTOR-dependent cellular compounds. Altogether, it seems likely that LARP1 holds a considerable potential as cancer therapeutic target and a key element of the translation machinery.

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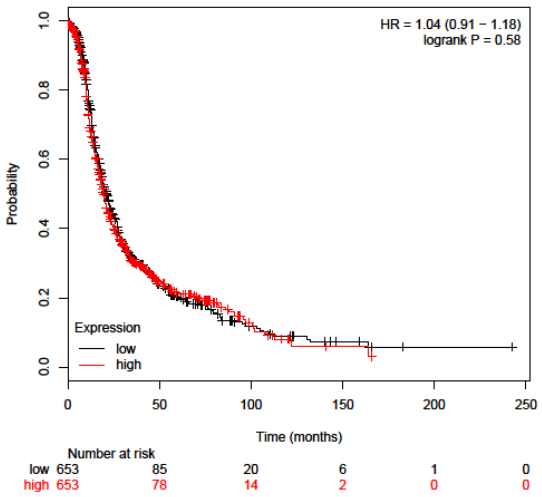
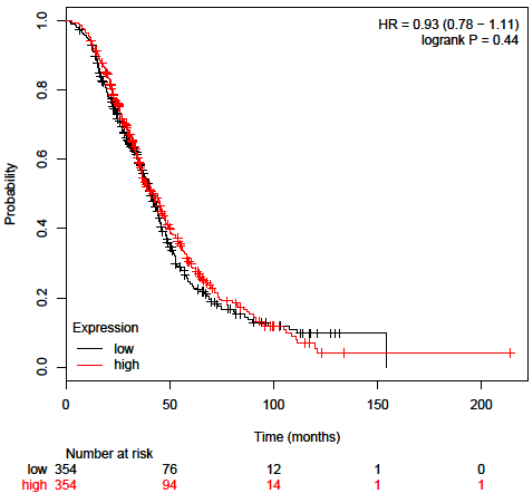
7 . A P P E N D I C E S

Appendices _ Kmplot

Ovarian cancer LARP1 PFS (n=1306/ 1307)

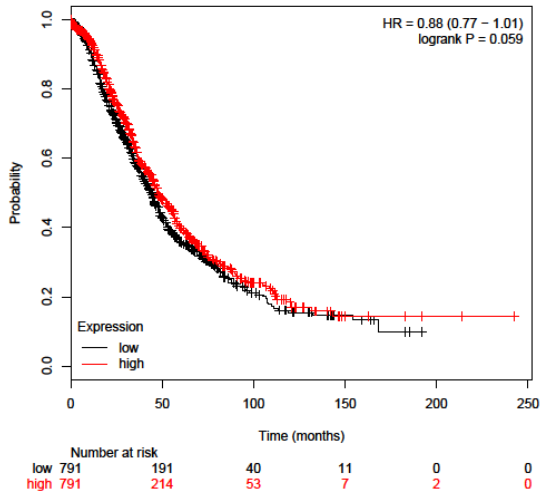


Breast can Ovarian cancer LARP1_PPS (n=708)



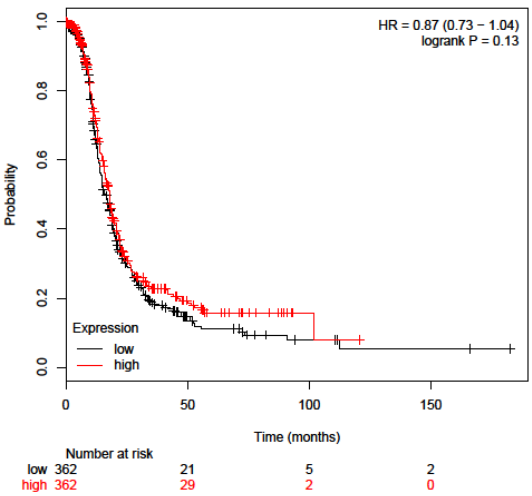
Ovarian cancer LARP1_OS

(n=1582)

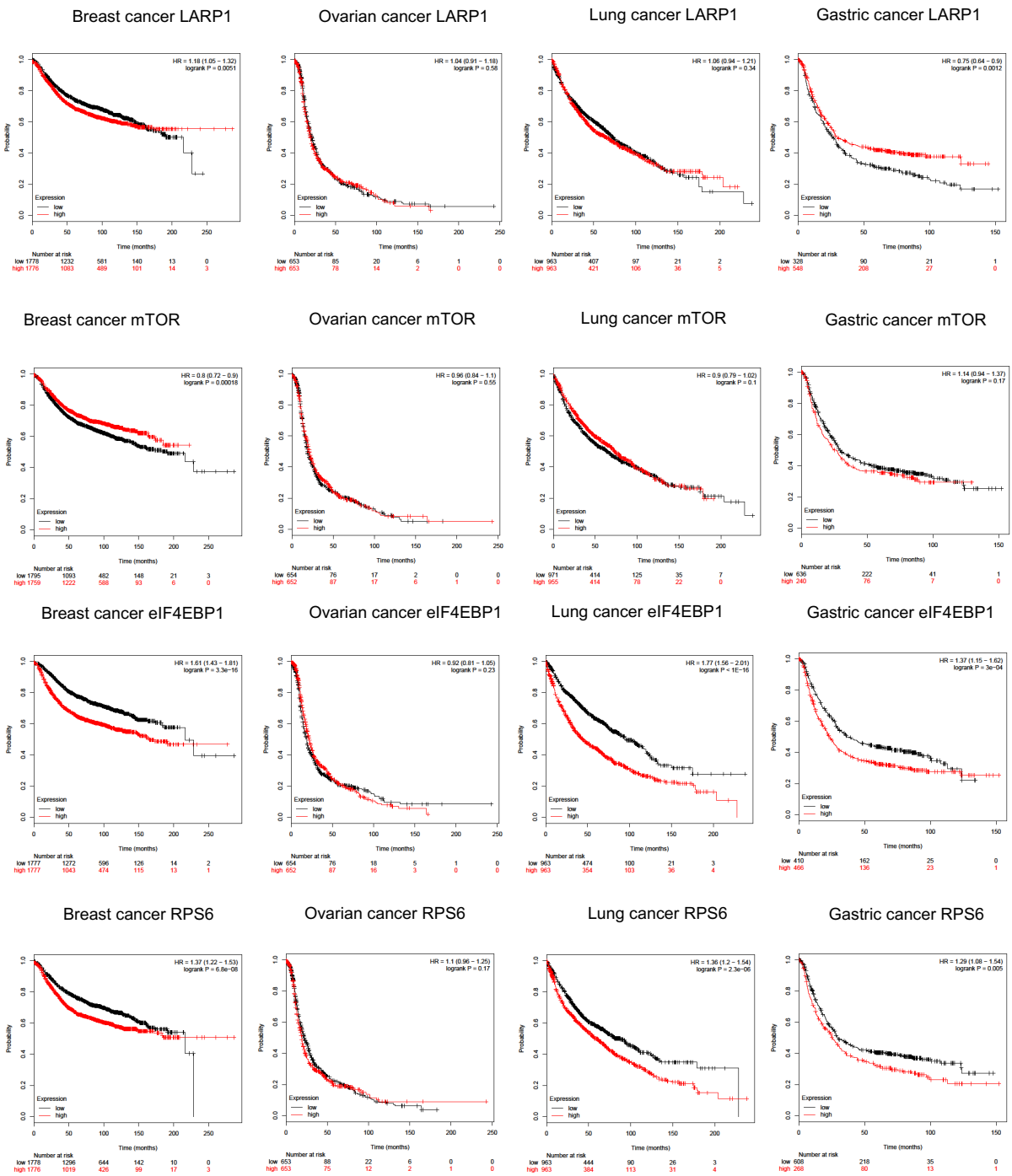


Ovarian cancer LARP1_PFS_Grade3_stage3-4

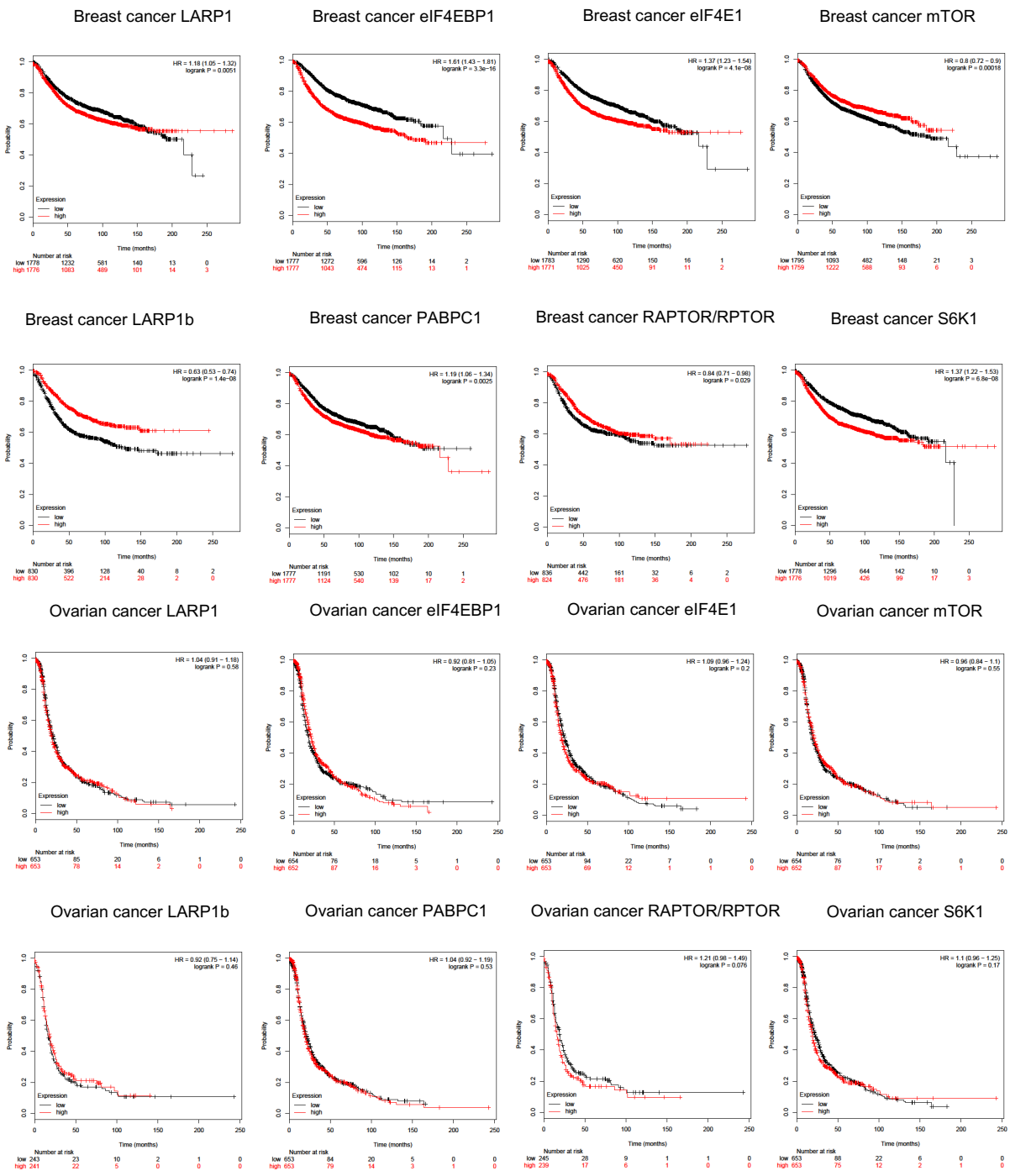
(n=1306 of 1307



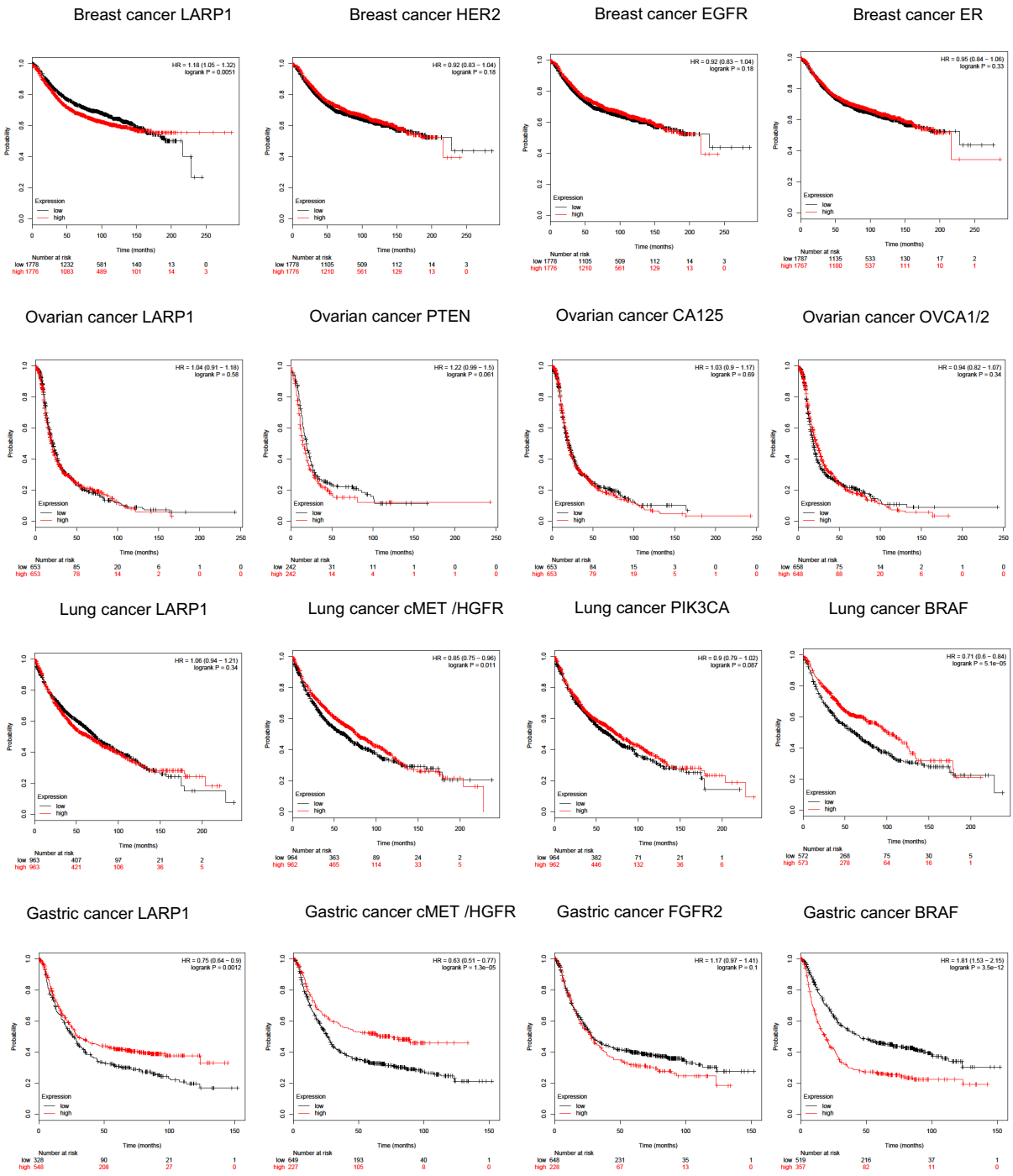
Appendix 1. Kaplan-Meier analysis comparing post-progression survival (PPS), Overall survival (OS) and Progression Free Survival (PFS). Ovarian cancer patients by LARP1 expression. Data available at www.kmplot.com



Appendix 2. Kaplan-Meier analysis of survival for cancer patients separated by the expression of different genes. Selected components of the mTOR pathway (mTOR, eIF4EBP1 and RPS6) are compared to LARP1. Data available at www.kmplot.com

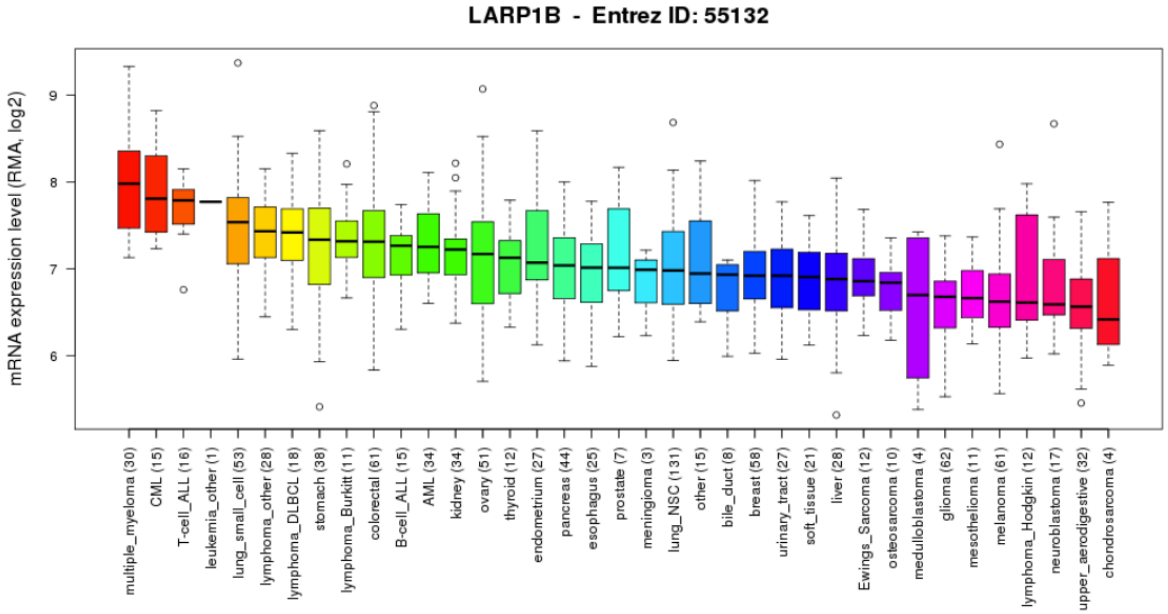


Appendix 3. Kaplan-Meier analysis of overall survival of breast and ovarian cancer patients separated by the mRNA expression of selected genes compared to LARP1 mRNA expression. Version 2015. Available at Kmplot.com

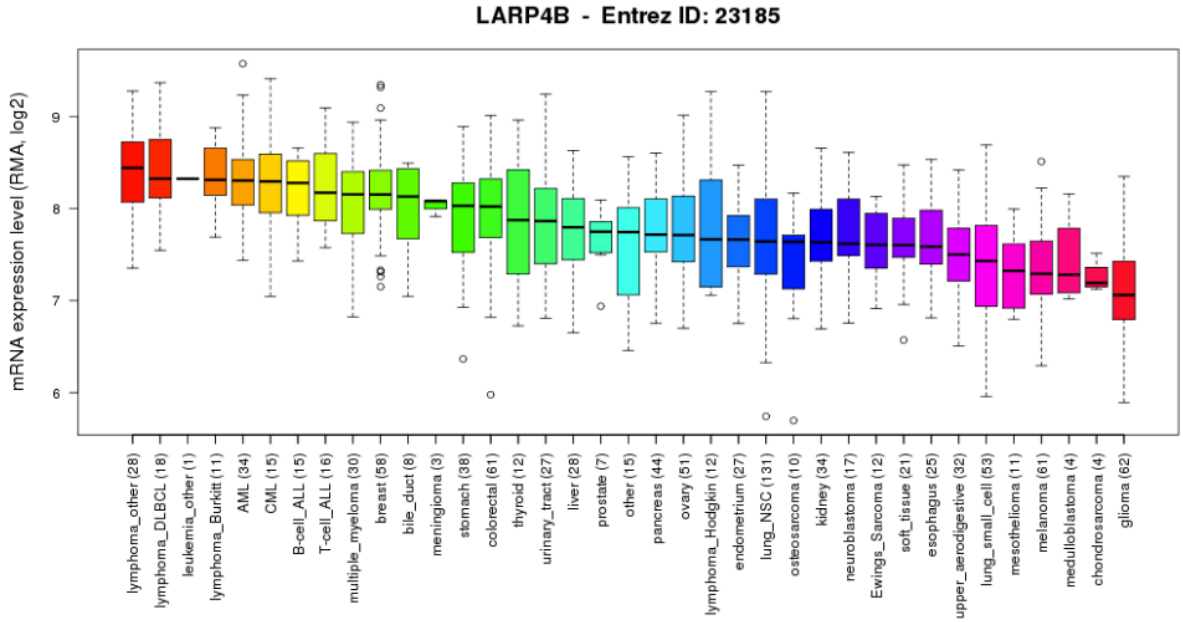


Appendix 4. Kaplan-Meier analysis of survival for cancer patients separated by the expression of different selected genes. Genes known to be altered or amplified in different cancers are compared to LARP1.

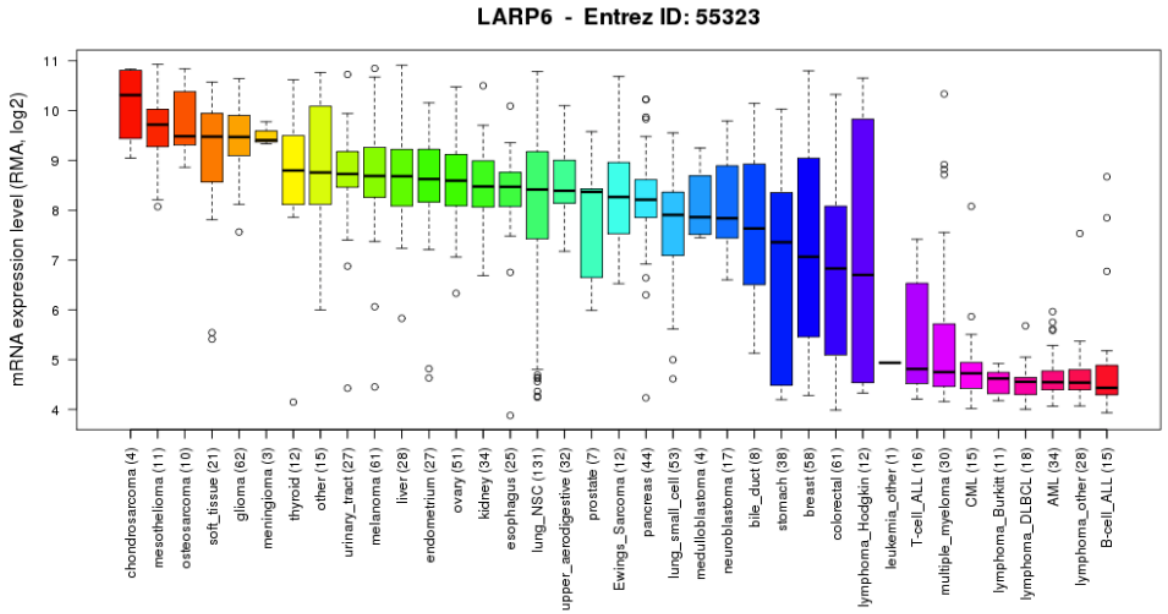
Appendices _ LARP1 mRNA expression



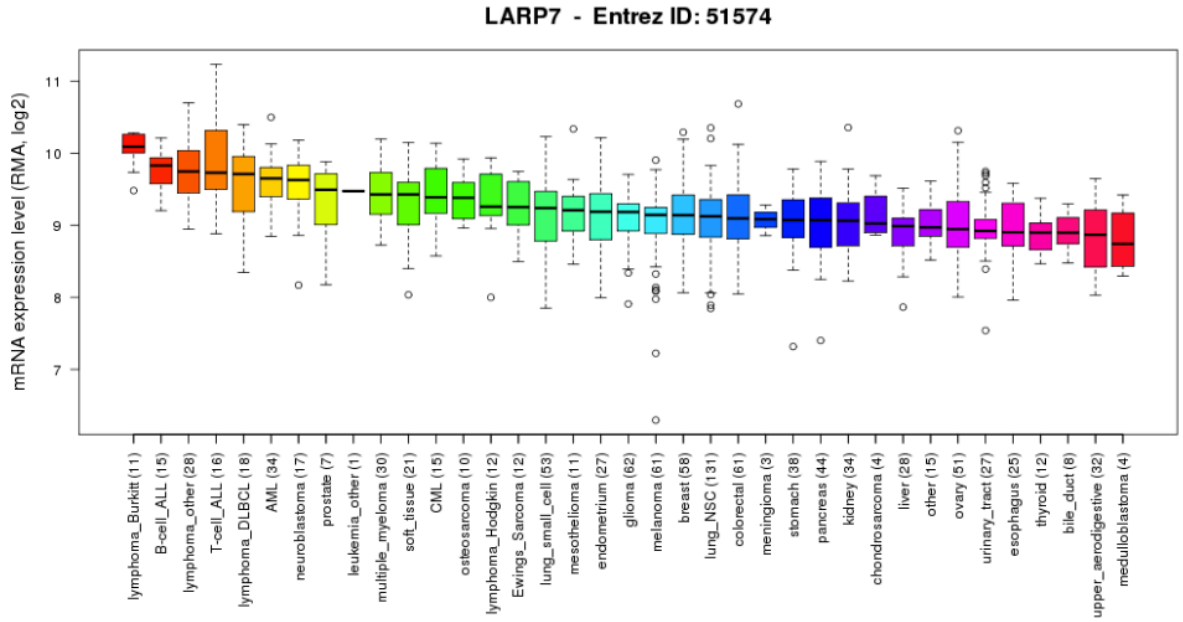
Appendix 5. LARP1b mRNA expression in different tumor cell lines. Data generated from the cancer cell line encyclopedia (www.broadinstitute.org/ccle/home).



Appendix 6. LARP4b mRNA expression in different tumor cell lines. Data generated from the cancer cell line encyclopedia (http://www.broadinstitute.org/ccle/home).

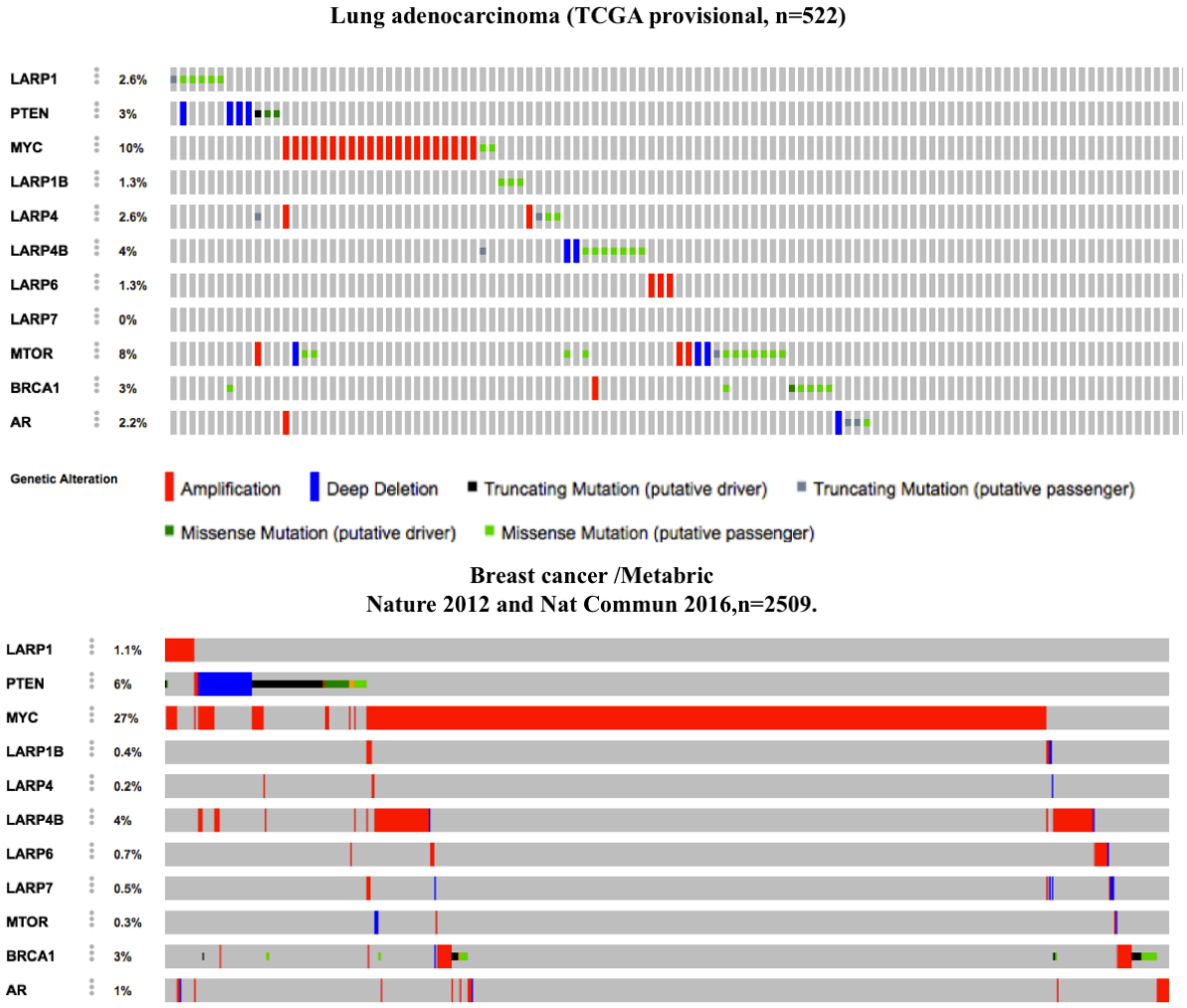


Appendix 7. LARP6 mRNA expression in different tumor cell lines. Data generated from the cancer cell line encyclopedia (http://www.broadinstitute.org/ccle/home).

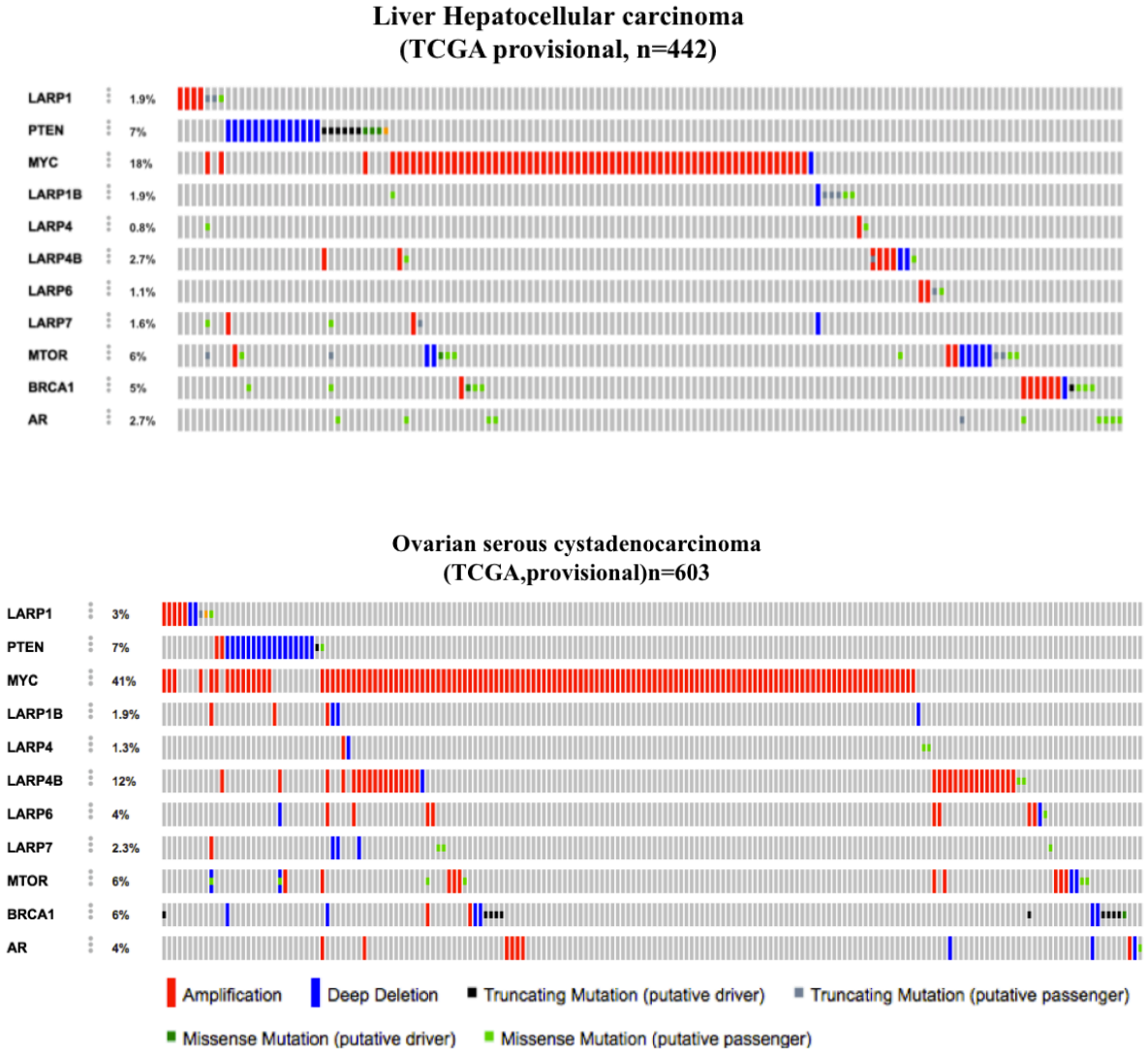


Appendix 8. LARP7 mRNA expression in different tumor cell lines. Data generated from the cancer cell line encyclopedia (http://www.broadinstitute.org/ccle/home).

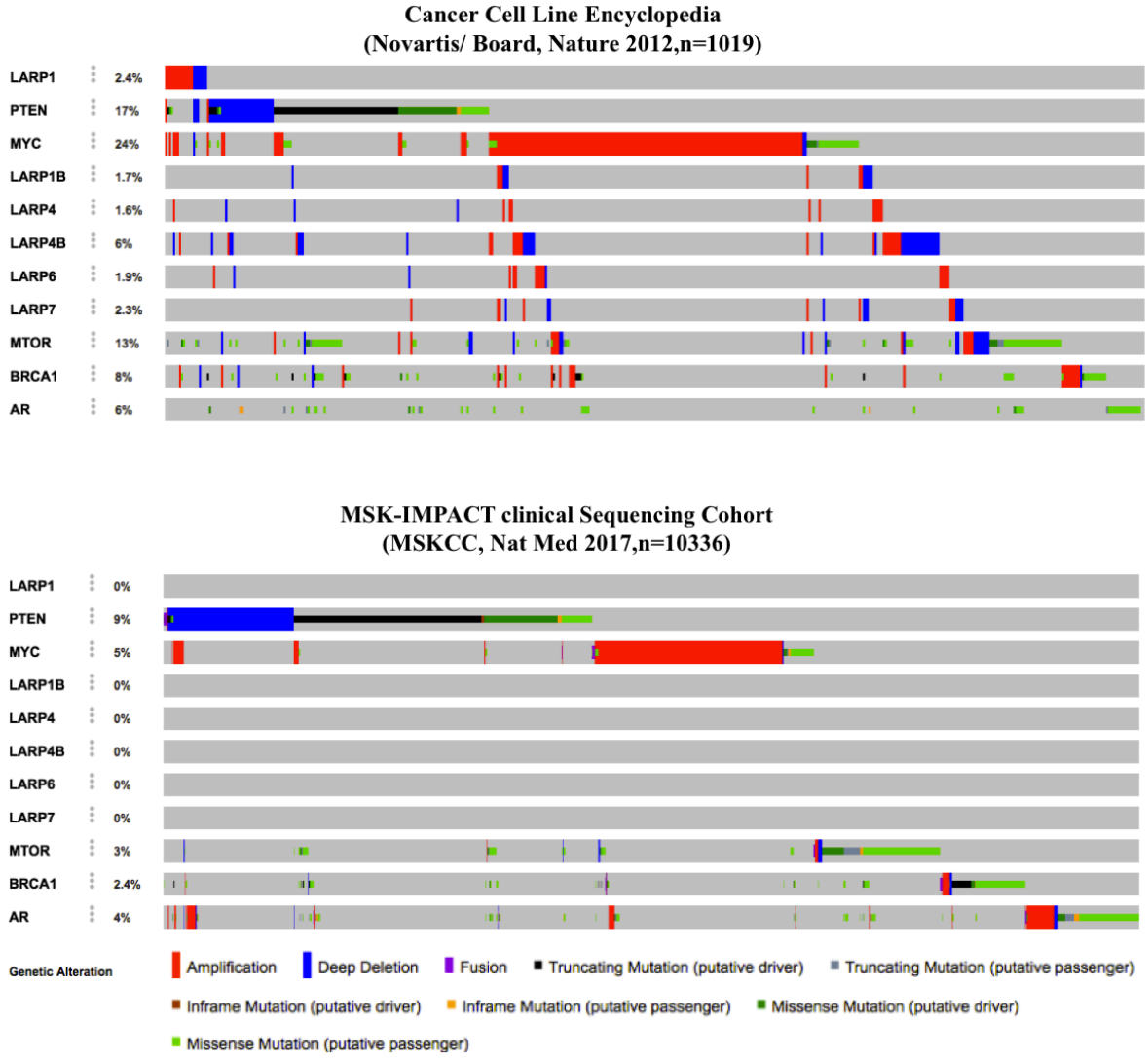
Appendices_LARP1 genetic alterations in cancer



Appendix 9. Genetic alteration frequency of selected genes in Breast and lung cancer patients. Grey bars along a vertical line represent the same sample interrogated for amplification (red), deep

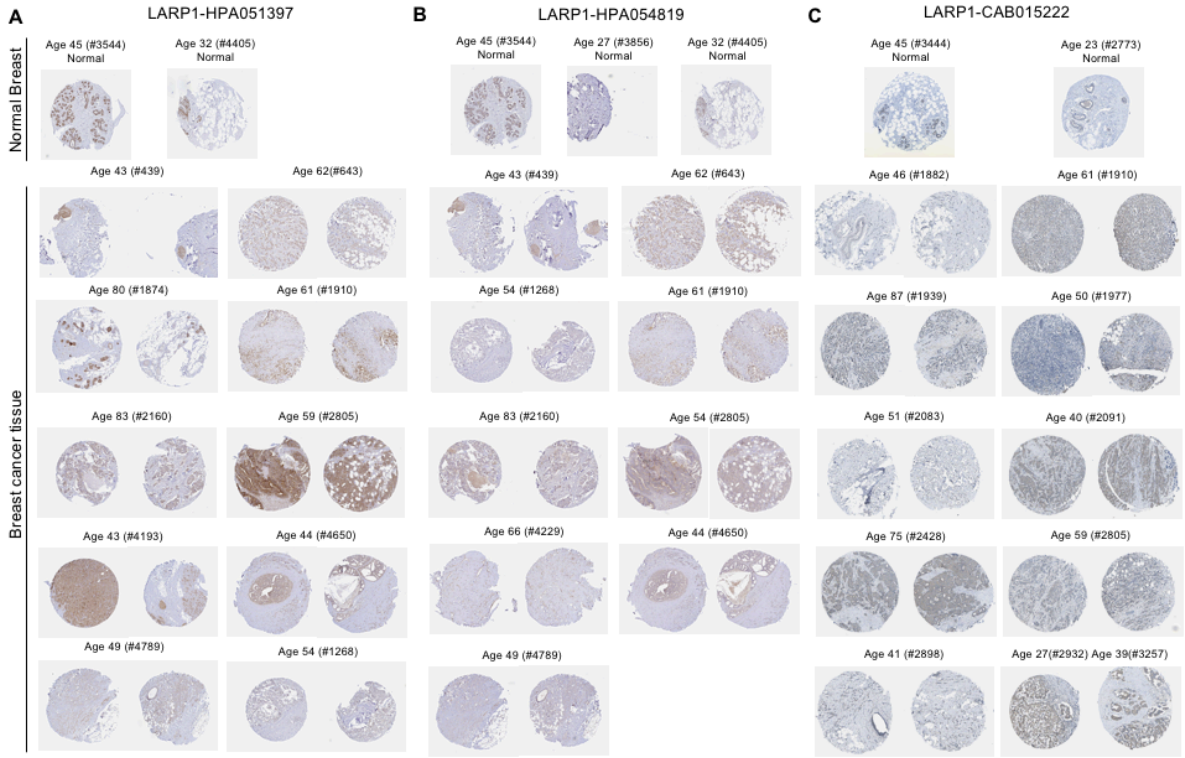


Appendix 10. Genetic alteration frequency of selected genes in liver and ovarian cancer patients. Grey bars along a vertical line represent the same sample interrogated for amplification (red), deep deletion (blue), missense mutation (green), truncating mutation (black) or in-frame mutation (brown).

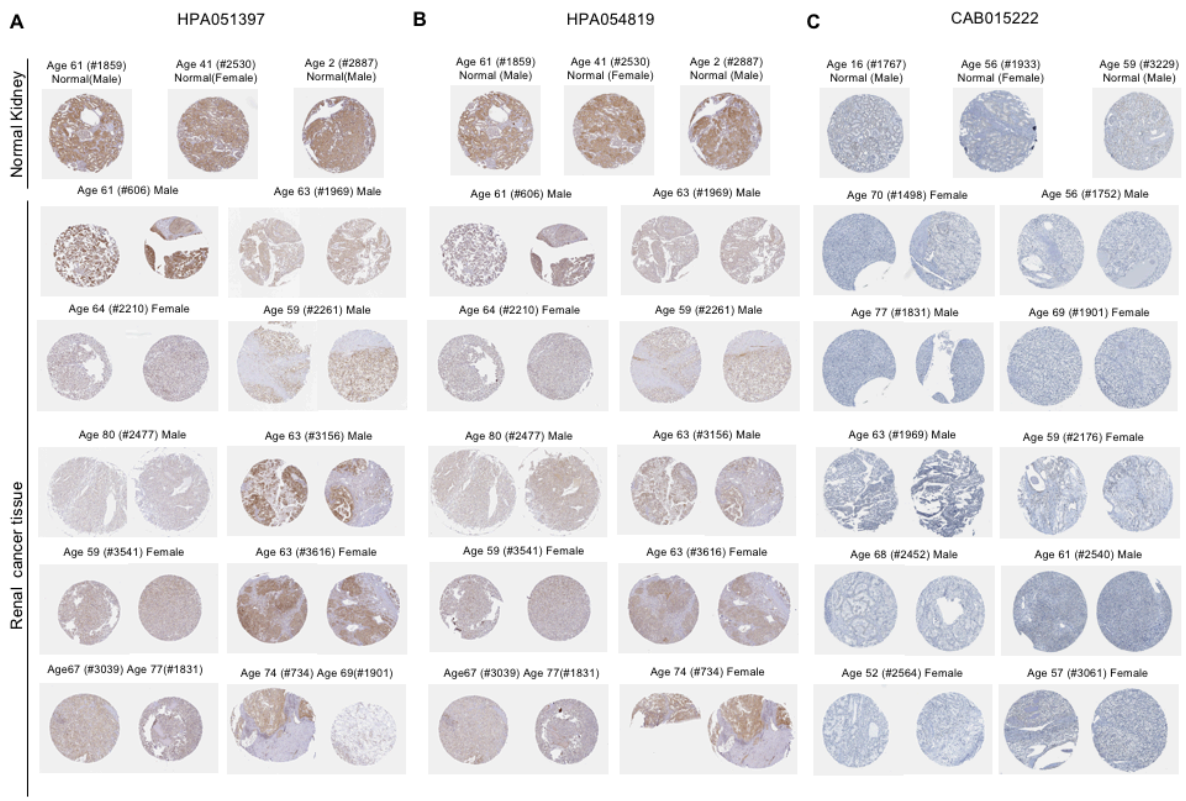


Appendix 11. Genetic alteration frequency of LARP1 and other selected genes in cancer cell lines and in cancer patients. Grey bars along a vertical line represent the same sample interrogated for amplification (red), deep deletion (blue), missense mutation (green), truncating mutation (black) or in-frame mutation (brown).

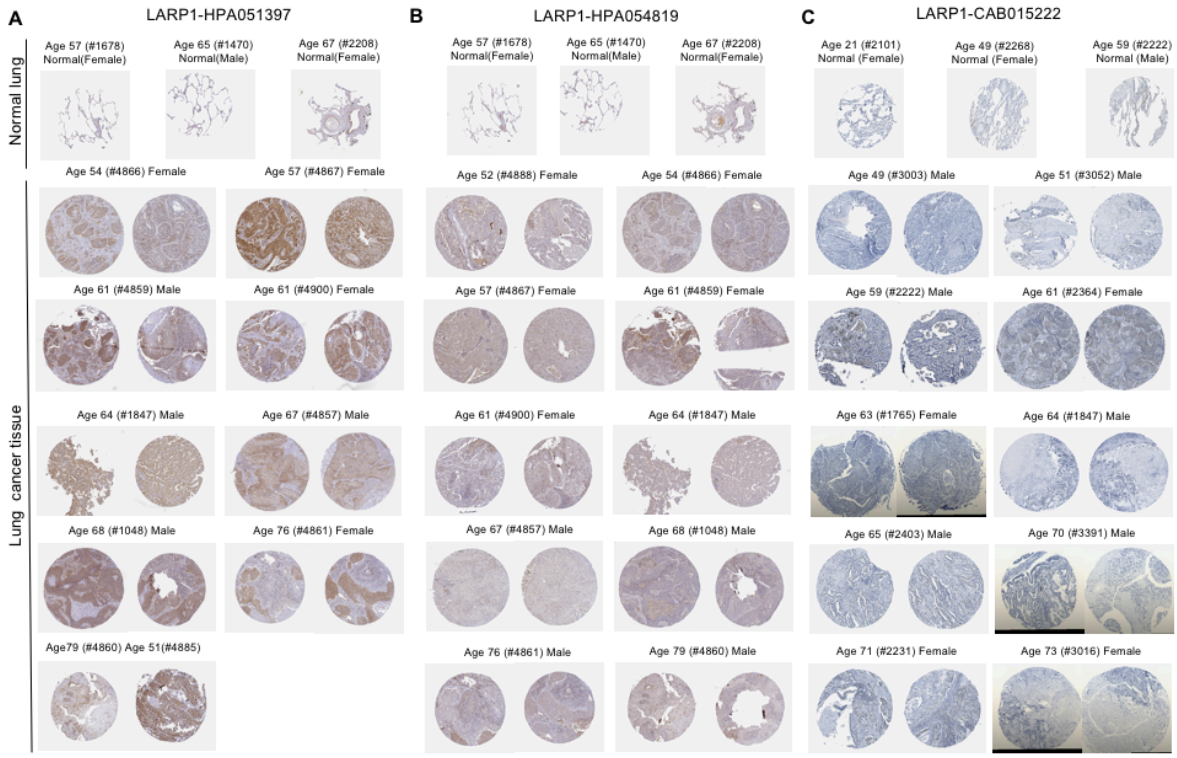
Appendices _ Human Protein Atlas



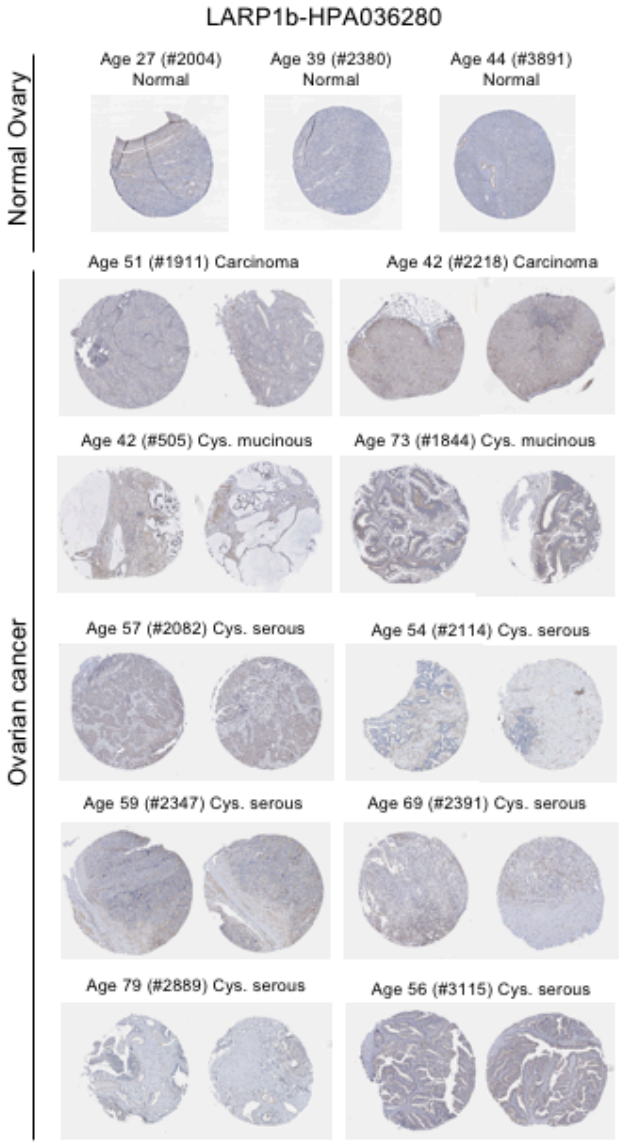
Appendix 12. Representative pictures of LARP1 protein expression in normal breast and breast cancer tissues with tissue microarray (TMA). A : staining with HPA051397 antibody. B: staining with HPA054819 antibody. C: staining with CAB015222 antibody. Data extracted and assembled from www.proteinatlas.org



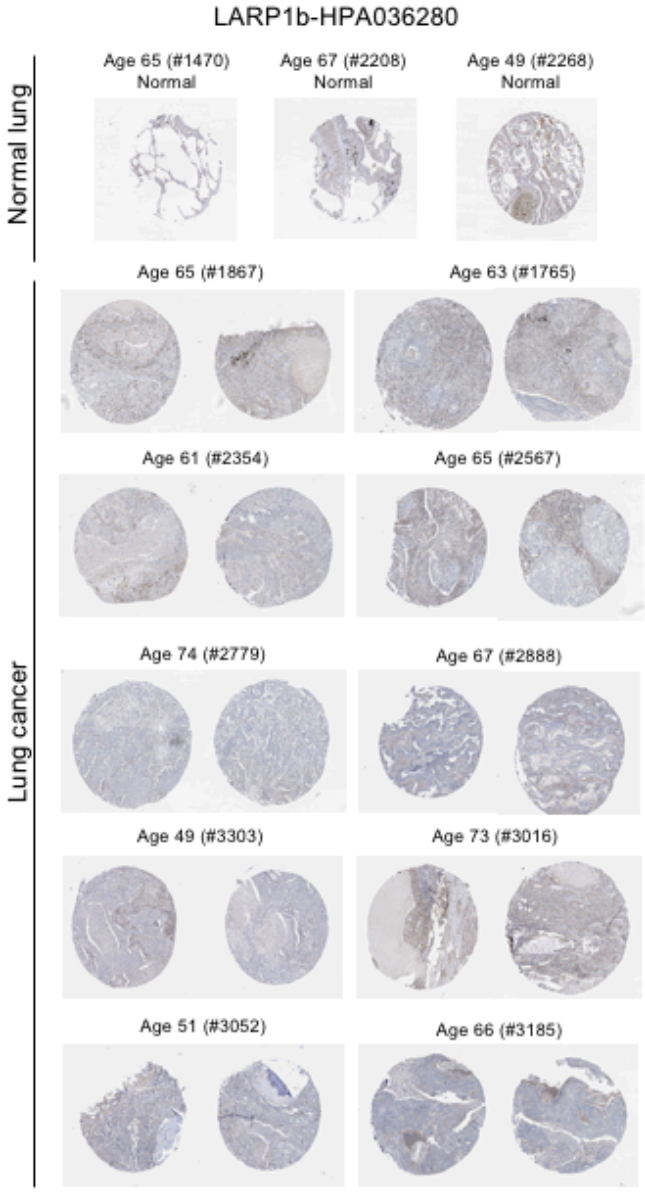
Appendix 13. Representative pictures of LARP1 protein expression in normal kidney and kidney cancer tissues with tissue microarray (TMA). A: staining with HPA051397 antibody. B: staining with HPA054819 antibody. C: staining with CAB015222 antibody. Data extracted and assembled from www.proteinatlas.org



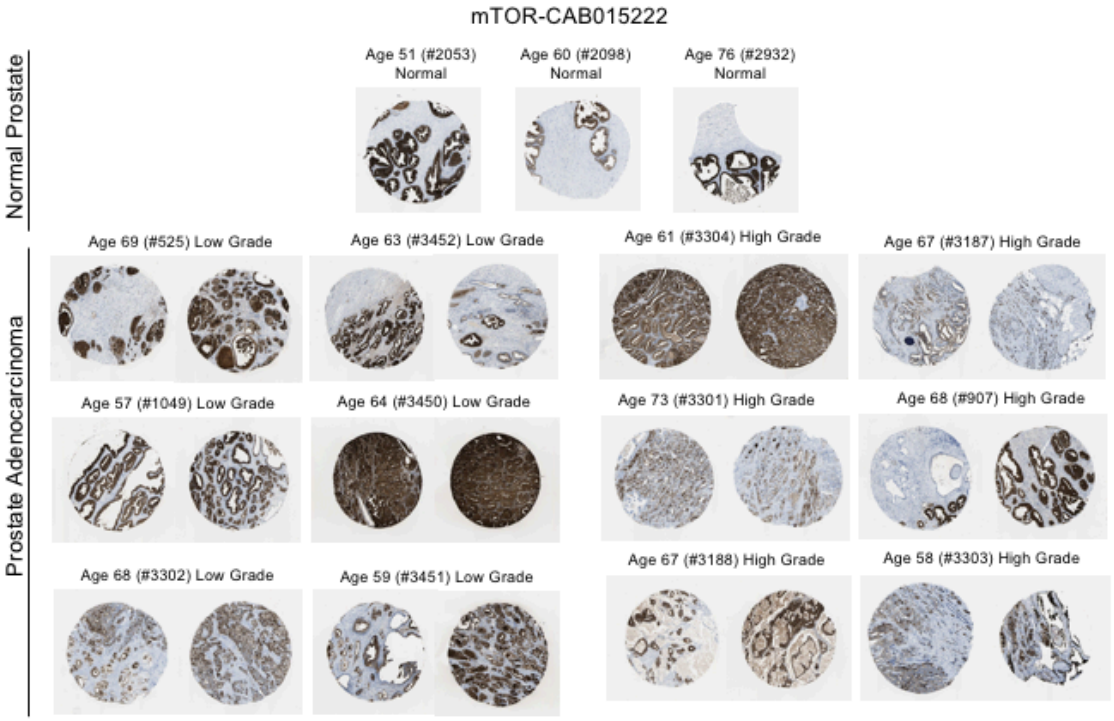
Appendix 14. Representative pictures of LARP1 protein expression in normal lung and lung cancer tissues with tissue microarray (TMA). A: staining with HPA051397 antibody. B: staining with HPA054819 antibody. C: staining with CAB015222 antibody. Data extracted and assembled from www.proteinatlas.org.



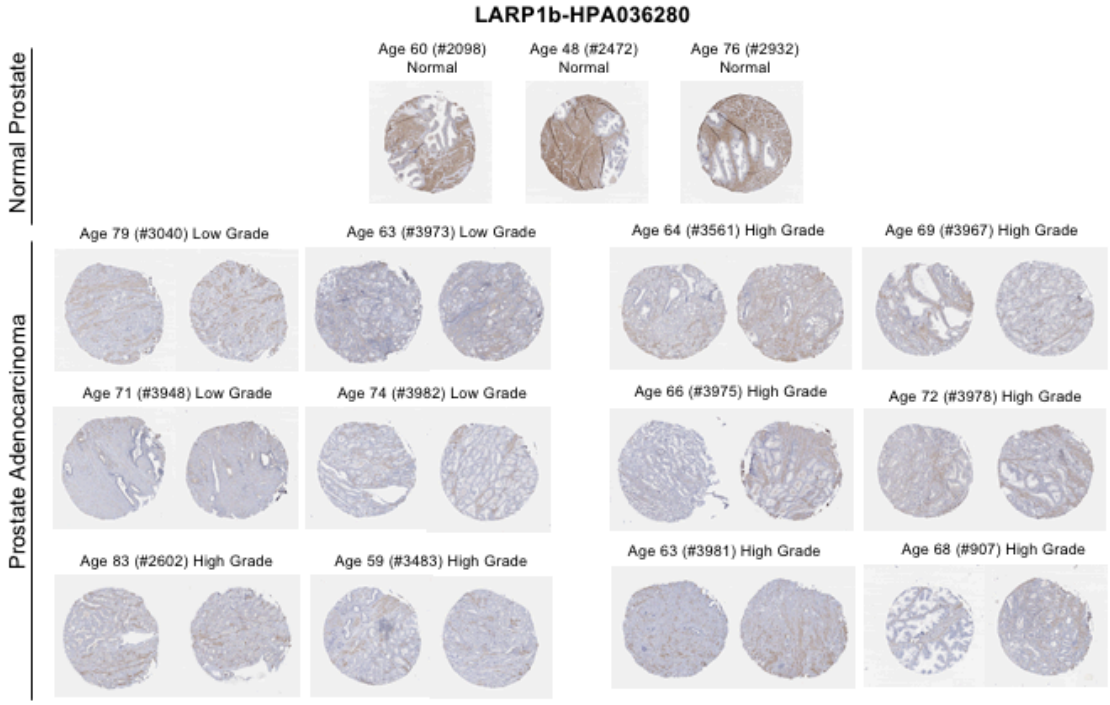
Appendix 15. Representative pictures of LARP1b protein expression in normal ovary and ovarian cancer tissues with tissue microarray (TMA). A: staining with HPA036280 antibody. Data from proteinatlas.org.



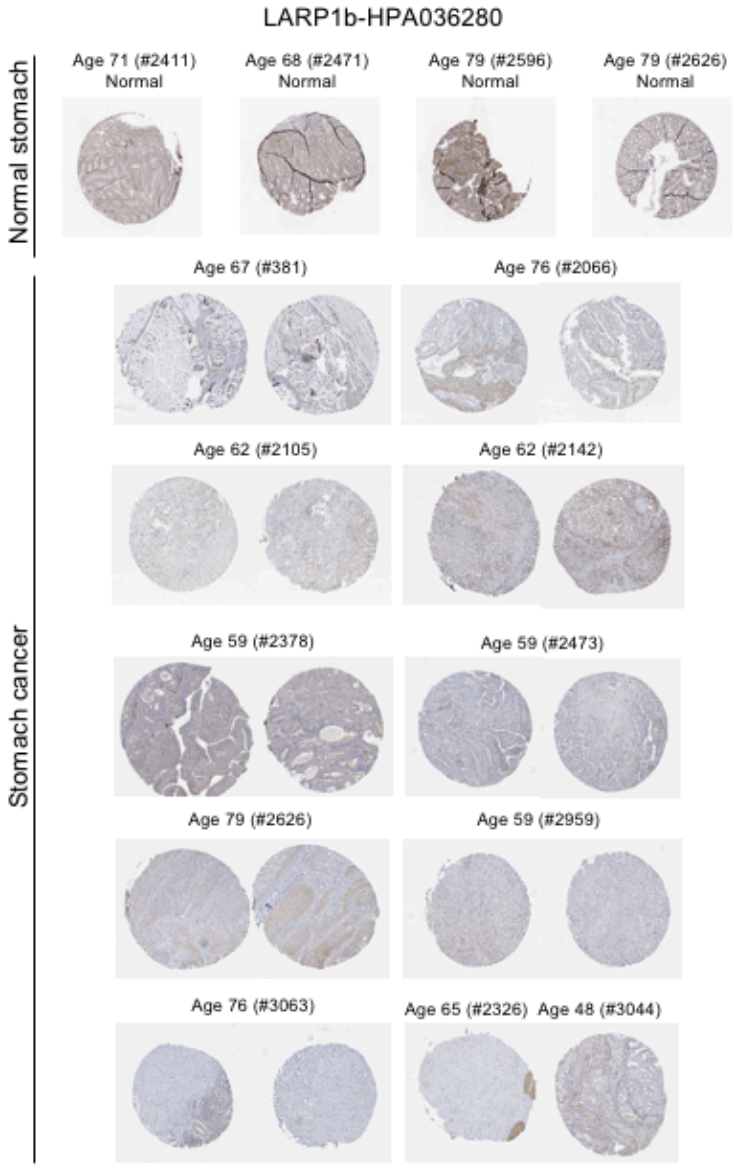
Appendix 16. Representative pictures of LARP1b protein expression in normal lung and lung cancer tissues with tissue microarray (TMA). A: staining with HPA036280 antibody. Data extracted and assembled from www.proteinatlas.org



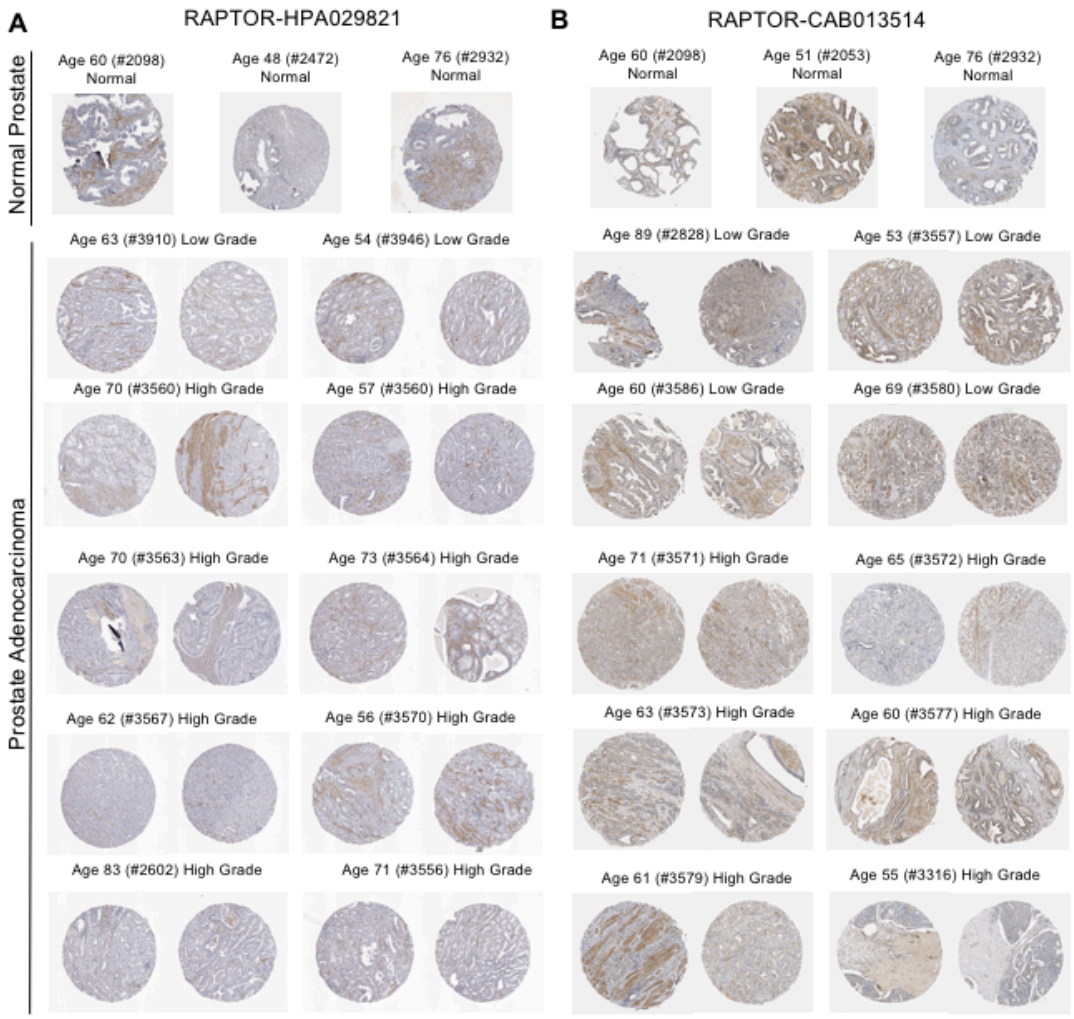
Appendix 17. Representative pictures of mTOR protein expression in normal prostate and prostate cancer tissues with tissue microarray (TMA). A: staining with CAB015222 antibody. Data extracted and assembled from www.proteinatlas.org



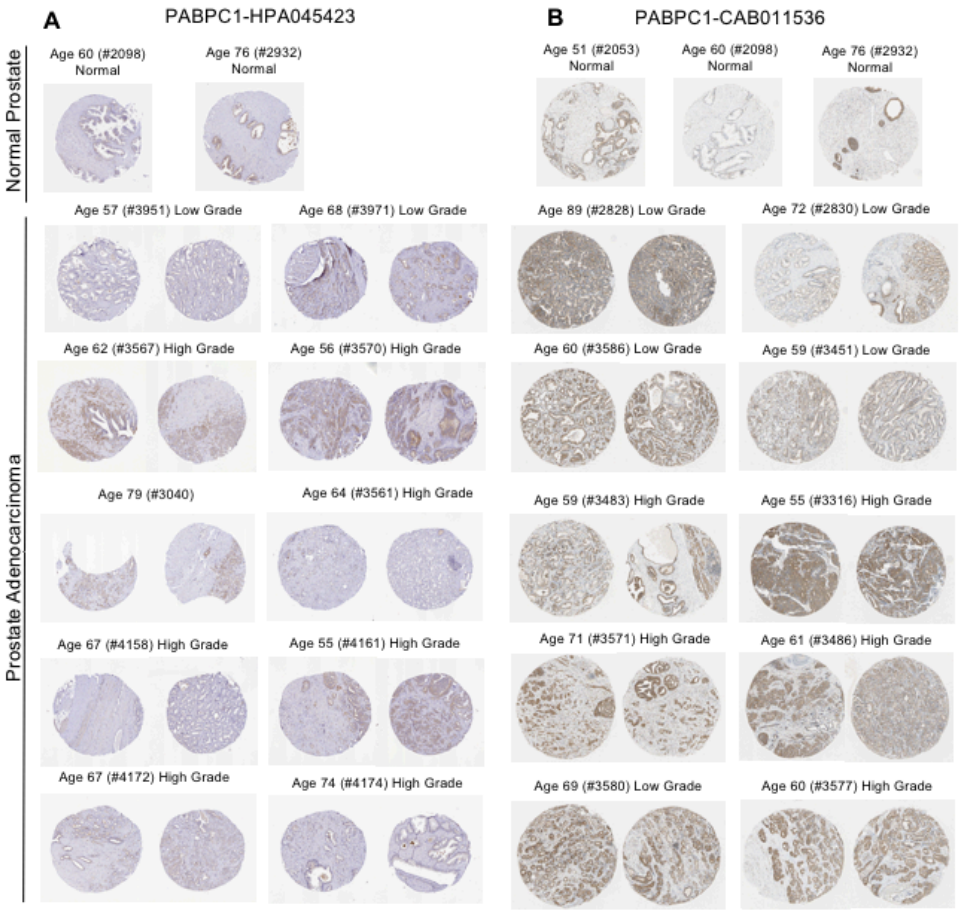
Appendix 18. Representative pictures of LARP1b protein expression in normal prostate and prostate cancer tissues with tissue microarray (TMA). A: staining with HPA036280 antibody. Data extracted and assembled from www.proteinatlas.org



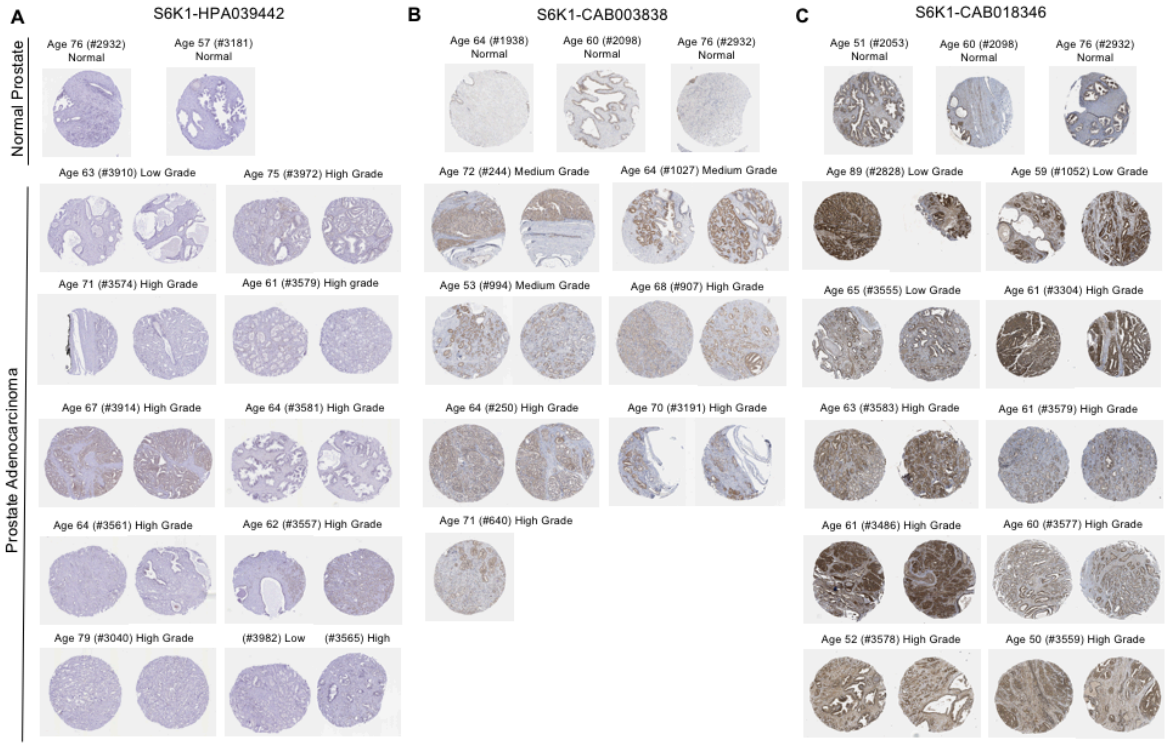
Appendix 19. Representative pictures of LARP1b protein expression in normal stomach and stomach cancer tissues with tissue microarray (TMA). A: staining with HPA036280 antibody. Data extracted and assembled from www.proteinatlas.org



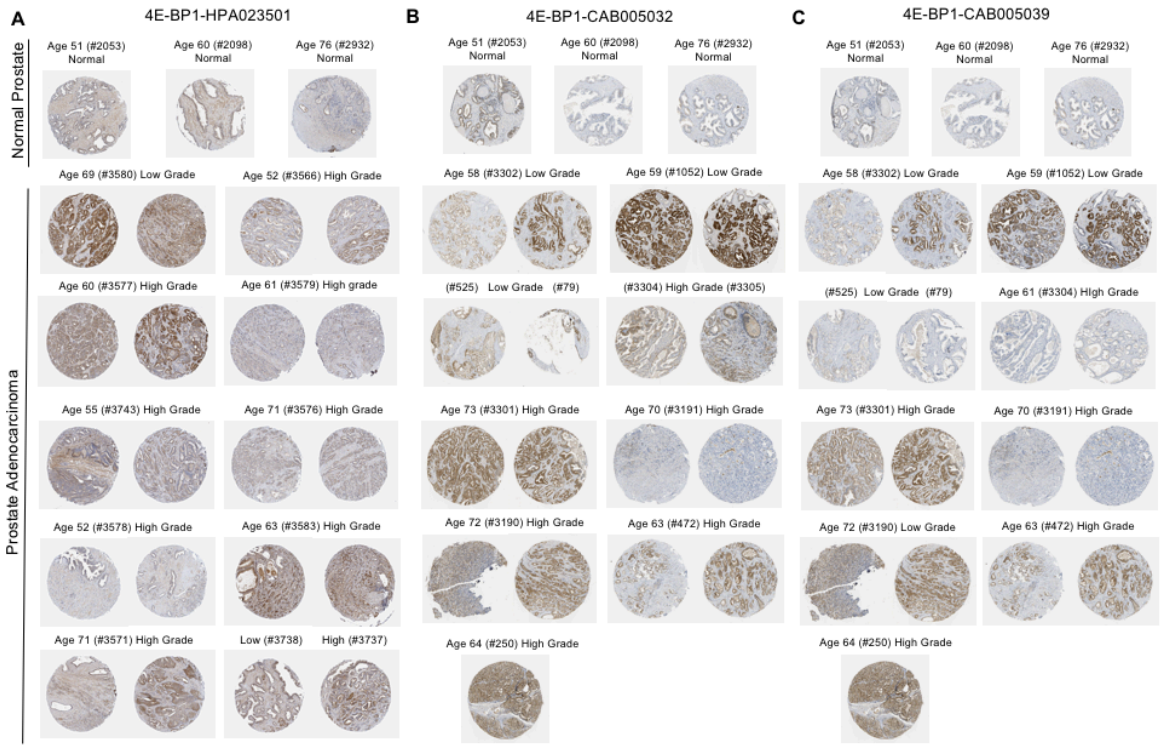
Appendix 20. Representative pictures of RAPTOR protein expression in normal prostate and prostate cancer tissues with tissue microarray (TMA). A: staining with HPA029821 antibody. B: staining with CAB013514 antibody. Data from proteinatlas.org



Appendix 21. Representative pictures of PABPC1 protein expression in normal prostate and prostate cancer tissues with tissue microarray (TMA). A: staining with HPA045423 antibody. B: staining with CAB011536 antibody. Data from proteinatlas.org



Appendix 22. Representative pictures of S6K1 protein expression in normal prostate and prostate cancer tissues with tissue microarray (TMA). A: staining with HPA039442 antibody. B: staining with CAB003838 antibody. C: staining with CAB018346 antibody. Data extracted and assembled from www.proteinatlas.org



Appendix 23. Representative pictures of 4E-BP1 protein expression in normal prostate and prostate cancer tissues with tissue microarray (TMA). A: staining with HPA023501 antibody. B: staining with CAB005032 antibody. C: staining with CAB005039 antibody. Data extracted and assembled from www.proteinatlas.org

8. CURRICULUM VITAE

Spoken languages: French, English and Russian

EDUCATION

Master of Science in Biochemistry 2015-

Faculty of Medicine, University of Ottawa

Master of Science in Life sciences 2012-2014

University of applied sciences Western Switzerland, Lausanne, Switzerland

Bachelor degree in chemical engineering and biotechnology 2007-2011

Tver state technical university, Tver, Russia

Secondary school Diploma 2004

Collège Saint Theophile. Main field: Scientific/ Biology and chemistry

SKILLS AND WORK EXPERIENCE

Quality control at the GMP oncolytic virus facility 2017

The Ottawa Hospital Research Institute (OHRI), Ottawa

- Certificate for Research Involving Humans (TCPS2)
- Perform assay qualification and release testing of Clinical samples
- Develop assay qualification protocols, execution of qualified assays, SOP development to support analytical assays for product release testing and stability studies

Research on cancer/apoptosis and translation control 2015-

Children's hospital of Eastern Ontario Research Institute (CHEO), Ottawa

- Immunofluorescence and immunohistochemistry
- Bioinformatic analysis :mRNA and protein expression
- Molecular biology techniques: PCR, molecular cloning, Western Blotting, …
- Tissue/cell culture

Research on epitope mapping with micro-array technology 2012 -2014

Institute of life technology, Molecular Diagnostic Research Group. HES-SO, Switzerland

- Epitope mapping and BARD1-gene .Breast and ovarian cancer in-vitro diagnostic
- Optimization of peptide Micro-array process for immunologic in-vitro tests.
- BARD1-protein as cancer biomarker

Peptide synthesis, MSc internship program 2012- 2013

Debiopharm laboratories, Valais, Switzerland

- Solid phase peptide synthesis using Fmoc chemistry
- Bioinformatics: Design of peptide libraries

- Peptide purification and analysis by UPLC/MS

Quality management and regulatory affairs 2012-2013

Hevs, Pixon Engineering, Lonza, Swissmedic, Switzerland

- Implementation of quality system in Life sciences industry (FDA and Swissmedic)
- Marketing license application and approval purposes (BLA; NDA, PMA, CE, …)
- Risk assessment (eg. Failure mode and effects analysis, FMEA)
- Validation processes (SOP and other related documents)
- Pharma process and principles of cGMP (product safety, pharma room / facility requirements, design concepts, contamination control etc.)
- ISO systems (eg. 9001 and 17025)

Quality excellence /Total quality management 2013

MLS BFH, Bern, Switzerland

- European foundation of quality management (EFQM)model and ISO 9004
- Continuous improvement process (CIP and PCDA model)
- Muda and Ishikawa model of QM
- FMEA (HACCP)
- KPI and 7W check list model

Professional training for engineering studies in Life sciences 2012-2013

School of Engineering, Sion, Switzerland

- Biotechnology: fermentation process, USP and DSP
- Basics of molecular biology
- Food microbiology

SOCIAL INVOLVEMENT AND INTEREST

- Volunteer at "Let's talk Science Canada"
- Volunteer at the CHEO Research Institute (summer 2015)
- Music: composition, piano, guitar ,Gospel and Gregorian Music.
- Sport : Football
- International and global exchange :Canada model UN(Ottawa),Model united nations(Geneva), Unesco Club(Paris, France), Seliger youth conference(Russia), Solidarité Jeunesse Francophone(D.R.Congo)
- Member of Journal Franco-jeunes(Québec), Journal Fracas (Université Laval), Journal fusion (Agence universitaire de la Francophonie)
- High interest in Bioethics