Imperial College London

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The role of the RNA binding protein LARP-1 in the post-transcriptional regulation of cisplatin resistant ovarian cancer

A thesis submitted for the degree of Doctor of Philosophy

Imperial College London

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DECLARATIONS

This thesis is submitted to Imperial College for the degree of Doctor of Philosophy. It has been composed in its entirety by me and has not been previously submitted either partially or in full for other degrees. The presented work represents my own original research carried out at the CRUK/Imperial Centre, in the Department of Surgery and Cancer, Imperial College London. I have acknowledged appropriately any assistance I have received in addition to that provided by my supervisors. Furthermore, I have clearly referenced the presence of all material I have cited from other sources.

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PUBLICATIONS/PRESENTATIONS ARISING FROM THIS THESIS

- *Protein interactions involving LARP1 in chemotherapy resistant ovarian cancer lines*.

  Poster presentation at the AACR: Translation in control of cancer, A New Frontier in Cancer Biology and Therapy, Oct 2016, San Francisco

  Obtained the AACR Scholar in Training award for meritorious abstract

- *The RNA binding protein LARP1 is a post-transcriptional regulator of cisplatin resistance in ovarian cancer.*

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ABSTRACT

Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy accounting for over 4000 deaths annually in the UK. Due to the lack of a validated screening tool, patients usually present with advanced disease and develop resistance to platinum-based chemotherapy. Resensitising resistant tumour cells to cisplatin remains an unmet clinical need. The RNA-binding protein LARP1 is highly expressed in ovarian cancer and its depletion by RNA interference (RNAi) was found to restore platinum sensitivity in cisplatin-resistant EOC cell lines showing a synergistic anti-tumour effect with cisplatin.

Using immunoprecipitation followed by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) in cisplatin sensitive (OVCAR3) and resistant (OVCAR8) ovarian cancer cell lines before and after cisplatin treatment, I identified PABP1, and YB-1 as strong, RNA-dependent LARP1 interactors in both cell lines. Upon cisplatin treatment, the interaction of LARP1 with YB-1 was preserved only in the resistant cell line and was further investigated as both proteins are known for promoting cisplatin resistance. In untreated OVCAR8 and OVCAR3 cells, LARP1 and YB-1 are predominantly cytoplasmic but accumulate in the nucleus upon cisplatin-induced genotoxic stress.

LARP1 and YB-1 are both in complex with, and regulate the mRNA transcripts of genes linked to cisplatin resistance such as the efflux ATPase pump ATP7B, the DNA damage binding protein 2 (DDB2) and the pro-survival factor BCL2. Both LARP1 and YB-1 act on their targets by regulating their mRNA abundance with LARP1 showing a greater effect.

Using the Surface Sensing of Translation (SUnSET) method, LARP1 was found to play a fundamental role in maintaining protein synthesis during genotoxic stress.

I conclude that LARP1, via its RNA-mediated interaction with YB-1, is a key post-transcriptional regulator of genes involved in pre-and post-target mechanisms of cisplatin resistance. Furthermore, it plays a vital role in preserving “de novo” protein synthesis and consequently cell survival during cisplatin induced genotoxic stress. Its diverse and fundamental functions make it a promising therapeutic target.

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ABBREVIATIONS

Abbreviation	Term
5'TOP	5' terminal oligopyrimidine tract
aa	Amino acid
CTD	C-terminal domain
EOC	Epithelial ovarian cancer
GO	Gene ontology
HGS	High grade serous
IP	Immunoprecipitation
LARP	La-related protein
MNase	Micrococcal Nuclease
mRBP	mRNA binding protein
mRNP	Messenger ribonucleoprotein particle
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
NTD	N-terminal domain
P-TEFb	Positive transcription elongation factor -b
PBS	Phosphate-buffered saline
PBST	Phosphate buffered saline with TWEEN
PIC	Pre-initiation complex
PLB	Polysome lysis buffer
Pol II/III	RNA-polymerase II/III
RBD	RNA-binding domain
RNA-Seq	RNA sequencing
RNP	Ribonucleoprotein particle
RT-qPCR	Real-time quantitative PCR
snRNA	small nuclear RNA
STIC	Serous tubal intraepithelial carcinoma
TBP	TATA- binding protein
TBS	Tris-buffered saline
TBST	Tris-buffered saline with TWEEN
UTR	Untranslated region

1 CHAPTER I – INTRODUCTION

1.1 CHAPTER ONE -ABSTRACT

1.2 OVARIAN CANCER

1.2.1 EPIDEMIOLOGY

Approximately 225,000 new cases of ovarian cancer are diagnosed worldwide with 7,400 cases in the UK [1, 2]. Epithelial ovarian, fallopian tube or primary peritoneal cancer, thereafter described as "ovarian cancer", is the second most common gynaecological malignancy in the UK after uterine cancer, but remains the most lethal accounting for 4,128 deaths annually [2]. UK and Russia are among the counties with the highest incidence of ovarian cancer, whereas China has the lowest [3]. Advances in integrated, subspecialized surgical approaches and the routine use of platinum-based chemotherapy regimens have led to an improvement in the 5-year survival figures over the last three decades, but it still remains relatively low at 46.5% [4-6]. Epithelial ovarian cancer (EOC) still remains more lethal than all the other gynaecological malignancies [2] Figure 1-1. The lack of a validated screening tool leads to the majority of patients (>80%) presenting with disseminated disease and approximately two thirds of these will develop resistance to cisplatin with poor prognosis [7]. These hurdles need to be overcome in order to improve survival rates as well as the quality of life in women diagnosed with ovarian cancer [8].

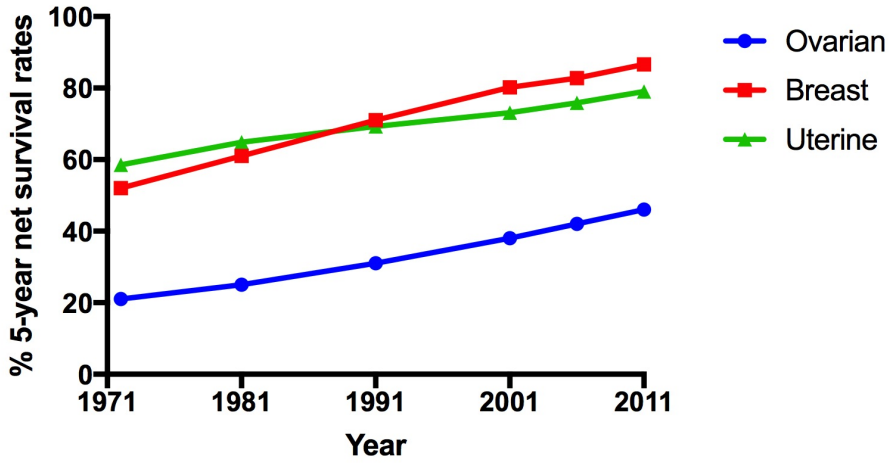


Figure 1-1 Five -year survival rates for ovarian, breast and uterine cancers in the UK.

Five-year survival trends for common gynaecological cancers over the last 4 decades. Data obtained from www.cancerresearchuk.org

1.2.2 OVARIAN TUMOUR CLASSIFICATION

Ovarian cancer is a rather heterogeneous disease which can be subdivided to several histological types, each one having their own distinct cells of origin, risk factors, molecular characteristics, clinical features and response to chemotherapy [9]. Epithelial ovarian cancers account for almost 90% of the diagnosed cases and based on their morphological characteristics they are subdivided into serous, endometrioid, mucinous and clear cell cancers[10]. Non-epithelial ovarian cancers account for 10% of the cases and include germ-cell and sex cord stromal tumours [10].

Epithelial ovarian cancers were for long considered and treated as a single entity. However, the fundamental differences in the molecular and phenotypical characteristics of each subtype urged a change in this approach. A new model for the classification has been proposed by Robert Kurman and le-Ming Shih, which categorises ovarian tumours in Type I and Type II [11]. Type I tumours include low grade serous, low grade endometrioid, clear cell and mucinous cancers. They usually present at a low stage as unilateral cystic ovarian masses and exhibit an indolent growth usually following a step–wise fashion from benign cystic neoplasms [12]. Type II tumours include high grade serous, high grade endometrioid cancers,

carcinosarcomas and undifferentiated carcinomas and present with papillary, glandular and solid appearances [11] . They exhibit a more aggressive behaviour than Type I tumours and present in advanced stage in most cases. Type II ovarian tumours display TP53 mutation in over 80% of the cases and have a distinct genetic mutation profile compared to Type I, which accounts for their phenotypical differences, and is summarised in Table 1-1 [13]. Overall type II tumours have a higher degree of genetic instability compared type I.

Table 1-1 Classification of epithelial ovarian cancer tumours

	Carcinoma	**Putative precursor**	**Genetic mutations**
Type I tumours	Low grade serous	Serous borderline tumour	KRAS, BRAF, ERBB2
	Low grade endometrioid	Endometriotic cyst	CTNNB1, PTEN, PIK3CA, ARID1A
	Clear cell	Endometriotic cyst	PIK3CA, ARID1A
	Mucinous	Mucinous cystadenomas and borderline tumours	KRAS
Type II tumours	High grade serous	Tubal and/or ovarian surface epithelium	TP53, PTEN, BRCA1/2
	High grade endometrioid	Unknown	TP53
	Carcinosarcoma	Unknown	Uknown
	Undifferentiated carcinoma	Unknown	Uknown

Adapted from Nik et al [14] with additional data from [13, 15].

1.2.3 RISK FACTORS

The incidence of epithelial ovarian cancer (EOC) is strongly correlated to age, with the majority of cases being diagnosed in post-menopausal women aged 65 years old and over [2, 16]. Apart from age, several risk factors have been linked to ovarian cancer and 21% of cases diagnosed each year in the UK are attributed to lifestyle and environmental factors. These mainly include: exposure to asbestos, tobacco smoking, hormonal replacement therapy (oestrogen only), talc-based powder (perineal use), X-radiation, gamma radiation obesity and nulliparity [17-19]. A family history of ovarian cancer increases the risk of the disease by 3 times but only 5-15% of the cases are inherited and the majority of these are linked to BRCA1/2 mutation and less frequently to Lynch syndrome [20, 21]. Germline BRCA1 and BRCA2 mutations are the most common genetic risk factors associated with ovarian cancer as well as with other malignancies such as breast (BRCA1 and 2), prostate (BRCA2), melanoma (BRCA2), pancreatic (BRCA2) and endometrial (BRCA1) cancer [21]. Ovarian cancer patients carrying BRCA mutations have better survival outcomes comparted to those with wild type status [22]. Lynch syndrome, also known as hereditary non-polyposis colorectal cancer, is characterised by the inheritance of germline mutations in genes of the mismatch repair system such as MLH1, PMS2, MSH2 and MSH6 [23]. It is associated with increased risk of ovarian, colorectal and endometrial cancers as well as urinary tract, small intestine, gastric and biliary tract cancers [23]. High grade serous ovarian cancers are most commonly associated with BRCA mutations, whereas endometrial and clear cell carcinomas are most common in Lynch syndrome [24, 25].

1.2.4 PRECURSOR LESIONS

The cell of origin of EOC has been long debated and it was initially thought that ovarian malignancies arise from the ovarian surface epithelium (mesothelium), with subsequent metaplastic changes leading to different histological subtypes [12]. However, the normal ovarian epithelium does not have characteristics that resemble such tumours. The different EOC subtypes are embryologically derived from Müllerian tissue and not mesothelium [12]. This resulted to a theory suggesting that ovarian malignancies originate from Müllerian -type tissue lined cysts located in paratubal and paraovarian locations [26].

More recent compelling evidence suggests that EOC do not arise directly from the ovary. Serous ovarian cancers are derived from the fallopian tube; endometrioid and clear cell tumours arise from the endometrium, while mucinous and transitional cell (Brenner) tumours come from metaplastic transitional-type epithelial tissue located in the tubal-mesothelial junction [27].

Dysplastic changes have been identified in the fallopian tubes but not in the ovaries of patients genetically predisposed to develop ovarian cancer [27]. Furthermore, potential fallopian tube precursor lesions, known as serous tubal intraepithelial carcinomas (STICs), are seen in over 70% of patients with sporadic high grade serous ovarian and peritoneal cancers [28, 29]. This supports the proposed hypothesis that STICs are probably the source of high grade serous ovarian cancer in both sporadic and hereditary cases [12]. This hypothesis was further supported with data coming from animal models. Knockdown in fallopian tube secretory epithelial cells of genes such as BRCA2, TP53 and PTEN, which are frequently mutated in HSG cancer, resulted in the development of STICs in mice [30].

The deeper understanding of the mechanisms underlying the development of ovarian cancer is of critical importance for the prevention and treatment of this devastating disease.

1.2.5 DIAGNOSIS

Ovarian cancer usually affects post-menopausal women in their mid 60s [6], who may present with ascites (fluid in the peritoneal cavity) and non-specific symptoms such as abdominal bloating, early satiety, fatigue, and/ or abdominal discomfort [31]. They may also develop shortness of breath due to increased diaphragmatic pressure, pleural effusions or pulmonary emboli [10]. The general and non-specific symptomatology of the disease leads to its delayed diagnosis as the symptoms may be mistakenly attributed to different conditions [31]. Consequently, the vast majority of cases are diagnosed in late stages of disease, which dramatically decreases their chance of being cured [32]. The disease stage at diagnosis is a major determinant of survival. Stage I is associated with 80-90% 5-year survival, stage II with 50-80% whereas survival falls to 30-50% for stage III and to 13% for stage IV [32]. Therefore, early detection of ovarian cancer is of paramount significance.

Patients with suspected EOC undergo pelvic and rectovaginal examination followed by transvaginal ultrasound and a serum CA125 test. Further radiographic imaging (such as CT, MRI or PET scan may also be necessary). CA125 is a glycoprotein, which can be raised in 90% of patients with advanced stage EOC, but only in half of those with stage I disease [33]. Although CA125 is a useful marker for monitoring response to treatment and disease recurrence [33], it cannot serve as a single diagnostic marker due to its low specificity and high false positive rates [34, 35] and it is used in combination with other diagnostic tests. Surgical resection of the mass is required and the diagnosis is ultimately confirmed upon histological examination of the obtained tissue. Disease staging is defined by the complete surgical and imaging evaluation and, along with the histopathological assessment, these will guide the consequent treatment [10].

Table 1-2 International Federation of Gynaecology and Obstetrics (FIGO) ovarian cancer staging

Reproduced with permission from [36].

FIGO stage	Description
I	Tumour confined to ovaries or fallopian tubes
IA	Tumour limited to one ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
IB	Tumour limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
IC	Tumour limited to one or both ovaries or fallopian tubes, with any of the following: IC1: surgical spill IC2: capsule ruptured before surgery or tumour on ovarian or fallopian tube surface IC3: malignant cells in the ascites or peritoneal washings
II	Tumour involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer
IIA	Extension and/or implants on uterus and/or fallopian tubes and/or ovaries
IIB	extension to other pelvic intraperitoneal tissues
III	Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes
IIIA	IIIA: Positive retroperitoneal lymph nodes only (cytologically or histologically proven): IIIA1(i) Metastasis up to 10 mm in greatest dimension IIIA1(ii) Metastasis more than 10 mm in greatest dimension IIIA2: microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes
IIIB	Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
IIIC	Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
IV	Distant metastasis excluding peritoneal metastases IVA: pleural effusion with positive cytology IVB: parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)

FIGO: International Federation of Gynecology and Obstetrics

1.2.6 SCREENING

Despite the critical need to diagnose ovarian cancer at an early stage, we are still lacking an effective screening test. As mentioned above, CA125 is not an efficient screening test as it is only increased in 50% of early stage cancers and can also be raised in benign conditions such as ovarian cysts, uterine fibroids, and infections [37, 38]. Furthermore, not all types of ovarian cancer secrete CA125. Increased levels are usually seen in HGSC, whereas non-serous subtypes are associated with lower levels [39] .

Two large randomised controlled trials investigating the use of multimodal screening strategies, including serial CA125 assessment, have been conducted. The Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening stud enrolled 78,216 women with age range 55-74 years, who were randomly assigned into two groups; either receiving annual screening (comprising CA125 for 6 years and transvaginal ultrasound for 4 years) or the standard care (no CA125 or ultrasound, but could receive bimanual examination with ovarian palpation). The study did not identify any mortality benefit for the group that underwent multimodal screening [40].

The UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) recruited 202,638 women of mean age 50-74 years who were randomly allocated in 3 groups receiving: i) annual multimodal screening with serum CA125 interpreted with the "risk of ovarian cancer" algorithm followed by ultrasound if needed; ii) annual transvaginal ultrasound and iii) no screening. Outcomes of this study showed no significant reduction in mortality across the cohorts. Further follow up of this study was deemed necessary to evaluate the efficacy and cost-effectiveness of routine ovarian cancer screening [41].

The addition of novel biomarkers such as HE4, CEA, VCAM-1 have been evaluated in combination with CA125 in multimarker screening assays. Although these have demonstrated better sensitivity, specificity remains low with no overall superiority to CA125

alone [42]. There is still an unmet need for a novel screening biomarker to facilitate early and accurate diagnosis of ovarian cancer.

1.2.7 TREATMENT

The mainstay of treatment for ovarian cancer consists of cytoreductive surgery followed by platinum-based chemotherapy. Standard debulking surgery comprises total abdominal hysterectomy (TAH), bilateral salpingo-oophorectomy (BSO), omentectomy, appendicectomy (in the case of mucinous histology) and, according to the extent of disease, may also involve peritoneal and diaphragmatic stripping, removal of lymph nodes and/or splenectomy [43].

Patients with advanced disease (stage IC-IV) or those with early stage disease who are sub-optimally staged (i.e without lymph node removal) or at higher risk of recurrence (stage 1C grade 2/3, any grade 3 or clear-cell histology) [44] benefit from adjuvant chemotherapy [45]. Women with early stage unilateral, well-differentiated and encapsulated disease (stage 1a, grade1) or those with comprehensively staged Ib, well or moderately differentiated (grade 1/2) disease [44] are adequately treated with surgery alone and do not require adjuvant chemotherapy. In advanced EOC, patients who have had a complete macroscopic surgical clearance (no residual visible disease) have a significantly increased overall survival (OS) and progression free survival (PFS) [46].

The cornerstone of chemotherapy treatment over the last three decades has been a combination of carboplatin [dosed by Area Under the Curve (AUC) 5 or 6] and paclitaxel 175 mg/m^2 administered intravenously on a three-weekly basis for six cycles [47]. The majority of patients tend to respond well to first line platinum-based treatment, however more than 70% will relapse within the first two years, requiring further surgical resection and/or chemotherapy [7]. After its first relapse, ovarian cancer usually return again in the majority of cases and tumours tend to become more chemo-resistant during each subsequent

recurrence [7]. A short platinum-free interval between relapses indicating a very poor prognosis [48]. According to the 5th Ovarian Cancer Consensus Meeting, patients are defined as ‘platinum-refractory’ if their ovarian cancer progresses during therapy or within one month after the last dose of chemotherapy; ‘platinum-resistant’ if progression occurs within one to six months of completing platinum-based therapy; ‘partially platinum-sensitive, if progression occurs between six and twelve months; and ‘platinum-sensitive’ if the interval is more than 12 months [49] .

1.3 CISPLATIN – A KEY CHEMOTHERAPEUTIC AGENT

Cisplatin (*cis*-diamminedichloroplatinum (II) -CDDP) was first described in 1845 [50] but its strong antiproliferative effects were only recognised in late the 1960s in E.coli cultures [51] and haematopoietic xenografts [52]. After extensive preclinical and clinical investigations, cisplatin obtained US Food and Drug Administration (FDA) approval for the treatment of testicular and bladder cancer in 1978 [53] . Over the last three decades the use of cisplatin has been expanded to a range of solid tumours and has become the backbone of treatment of ovarian, lung and colorectal and head and neck cancers leading to significant progress in their management [54-56]. Despite the improvement in treatment outcomes that cisplatin based chemotherapy brought, only a relatively small proportion of patients achieve a durable response. Cisplatin has shown remarkable efficacy against testicular cancer, with the majority of patients achieving complete and sustained remission [57, 58]. On the other hand, the majority of ovarian cancer patients (particularly those with an advanced disease) experience an initial response to treatment before their disease relapses again becoming chemotherapy resistant [7]. This also applies to patients with lung, prostate and colorectal cancer as a significant proportion includes tumours which are intrinsically resistant to cisplatin [59]. The side effect profile of cisplatin is wide and includes mild to moderate neurotoxic, cardiotoxic and nephrotoxic symptoms [60]. In an effort to minimise its side effects while maintaining its therapeutic efficacy, two additional derivatives of cisplatin have been developed and licensed by the FDA for use in solid malignancies: carboplatin and oxaliplatin [61, 62]. Carboplatin obtained an FDA approval for use in ovarian cancer patients [61] and shares a similar mechanism of action with cisplatin and comparable efficacy, but causing less nephrotoxic and neurotoxic side effects [63]. Oxaliplatin entered clinical practice in 2002 in combination with 5-fluiorulacil (5-FU), both components of the FOLFOX regimen used for the treatment of colorectal cancer [62, 64]. Despite having distinct pharmacological and immunological

properties from cisplatin [65, 66], a significant number of cisplatin resistant tumours are also resistant to oxaliplatin and as well as to other chemotherapeutics with different mechanisms of action [67]. This reflects the complexity of the mechanisms underpinning cisplatin resistance.

1.3.1 CISPLATIN MECHANISM OF ACTION

Structurally, cisplatin is an inorganic neutral, square-planar complex consisting of two chloride groups and two relatively inert amine ligands. It remains chemically inactive until one or both of its chloro-groups are replaced by water molecules [68, 69]. Cisplatin enters the cytoplasm primarily via copper transporters located in the plasma membrane. Several transporters have been implicated in facilitating its intracellular entry including the Na+, K+-ATPase [70] as well as members of solute carrier (SLC) transporters [71] and particularly copper transporter 1 (CTR1) [72, 73]. Once in the cytoplasm, the concentration of chloride ions fall to ~ 2-10 mM as compared to the ~100mM found at the extracellular space and results in its spontaneous "aquation" and loss of one, or both of the *cis* chloro groups [69]. The mono- and bi-aquated forms of cisplatin have electrophilic properties and form covalent bonds with methionine and also with cysteine containing polypeptides such as reduced glutathione (GSH) and metallothioneins [74]. This interaction has a binary effect. Firstly, this promotes the establishment of oxidative stress, via the depletion of reducing equivalents from the cytoplasm, which may cause direct cytotoxity, or DNA damage [75, 76]. On the other hand it can act as a cytoprotective buffer, as the active CDDP gets inactivated [77].

The aquated CDDP forms covalent bonds with nucleic acids and proteins creating adducts [74, 78]. Cisplatin binds to both nuclear and mitochondrial DNA, particularly to the N7-sites of the DNA purine bases and forms inter- and intrastrand crosslinks [79-81]. If the extent of the DNA damage is limited, these lesions are recognised and removed by a number of repair pathways in the context of a temporary cell cycle arrest in the S and G2 phases of cell cycle

[82]. Proteins belonging in the nucleotide excision repair (NER) pathway [83, 84] as well as the mismatch repair (MMR) complex [85] play a predominant role in the removal of DNA adducts [83, 85, 86]. In the event of irreparable DNA damage, cells either undergo a permanent cell cycle arrest (cellular senescence) [87], or apoptosis [88]. Apoptosis is mediated through the activation of the ataxia telangiectasia and Rad 3 – related protein (ATR), which then activates the checkpoint kinase 1 (CHECK1), resulting in the phosphorylation of the tumour suppressor protein p53 on serine 20 which promotes its stabilisation [86, 89-91]. Activated p53 eventually exerts its pro-apoptotic function through a cascade of events which promote widespread mitochondrial outer membrane permeabilisation (MOMP) [92, 93].

The cytotoxic properties of cisplatin are mediated not only via its nuclear genotoxic activity but also through its cytoplasmic action [77, 81]. Although not yet fully elucidated, several potential mechanisms that underpin the cytoplasmic cytotoxic properties of cisplatin have been suggested [94]. Cisplatin promotes the establishment of oxidative stress through the accumulation of reactive oxygen species (ROS) and nitric oxide (NO), which not only potentiates its genotoxocity but also directly results in the opening of the permeability transition pore complex (PTPC) [95, 96]. Furthermore, it facilitates the transduction of signals that stimulate mitochondrial outer membrane permeabilisation (MOMP) via BAK1, the voltage dependent anion channel 1 (VDAC1) and BAX proteins [97] . Cisplatin has also been associated with the he establishment of endoplasmic reticulum (ER) stress response [98, 99]. Apart from DNA adducts, it has been found that cisplatin also forms RNA adducts, and accumulates to a greater extent in RNA than DNA [78, 100]. However, the exact implications of this interaction to cytotoxicity are not yet well described. The mechanisms underlying the cytotoxic effects of cisplatin are summarised in Figure 1-2 [94].

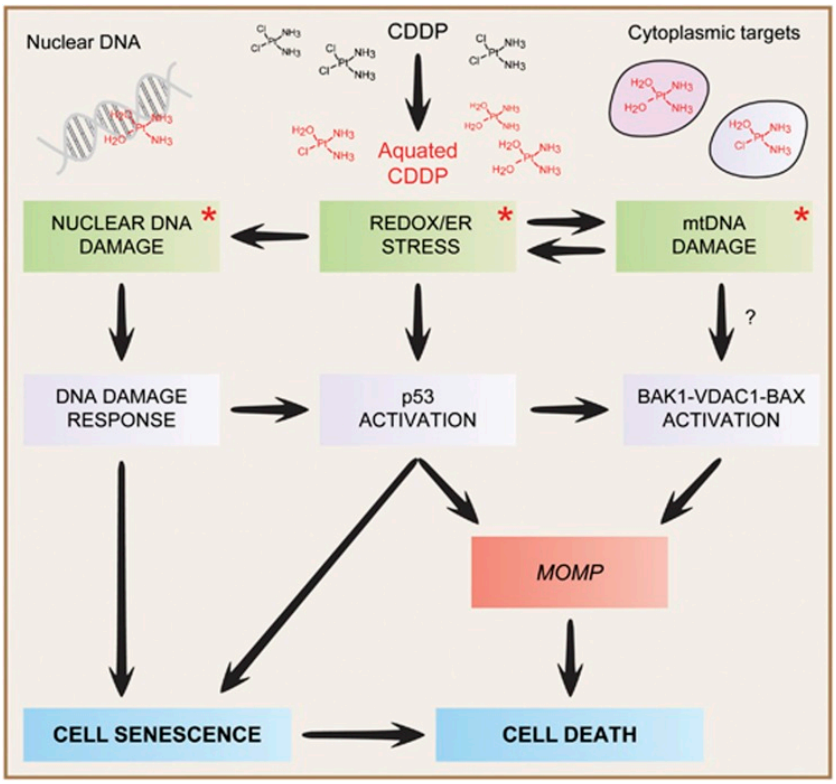


Figure 1-2 Overview of the mechanisms underpinning the cytotoxic effects of cisplatin

Figure reprinted with permission from Galluzzi et al (2014) [94]

Cisplatin (CDDP) is a compound with a square planar geometry composed by a doubly charged platinum ion surrounded by two NH3 and two Cl ligands. Cisplatin exerts its action via cytoplasmic and nuclear effects which contribute to its cytotoxic activity. CDDP molecules get aquated upon entering the cytoplasm due to the significant decrease in the chloride concentration [69]. The aquated cisplatin binds to nuclear DNA, on N7 sites of the purines, creating inter and intra-strand DNA adducts and consequently activating the DNA damage response leading to cell death. Apart from nuclear DNA, the aquated CDDP also binds mitochondrial DNA (mtDNA) as well as with cytoplasmic "scavengers" with nucleophilic properties such as reduced glutathione (GSH) and metallothioneins. Such interactions result to: 1) the establishment of oxidative and endoplasmic reticulum (ER) stress; 2) activation of cytoplasmic p53; 3) transduction of MOMP-stimulatory signals via BAK1-VDAC1-BAX1 pro apoptotic factors [94]. [MOMP: mitochondrial outer membrane permeabilisation; VDAC1: voltage-dependent anion channel 1]

1.3.2 MECHANISMS OF CISPLATIN RESISTANCE

The efficacy of chemotherapeutic agents in promoting cell apoptosis can be compromised by a number of factors which contribute to the development of "resistance". These involve: i. drug pharmacokinetics; ii. tumour micro-environment and iii. Cancer cell- specific characteristics [7].

In order for a chemotherapeutic drug to exert its cytotoxic activity a certain intratumoural concentration needs to be reached. This can be affected by a number of parameters which vary among patients due to genetic variation and include: first-pass drug metabolism, pro-drug conversion to active metabolites, renal clearance and vascularity of the tumour [101, 102].

The tumour micro-environment is another important factor affecting the cancer cell sensitivity. A characteristic example is the hypoxia-induced resistance to radiotherapy and chemotherapy due to an increase in the number of cancer cells undergoing cell cycle arrest as a response to hypoxia [103, 104]. The structural configuration of the tumour also affects the drug efficacy with *in vitro* studies demonstrating that cells growing as monolayers have different sensitivity to chemotherapy than spheroid cultures [105].

There are variations in the mechanisms that each tumour cell type employs to overcome chemotherapy. Drug resistance could be the outcome of progressively acquired somatic mutations or even epigenetic changes occurring in the tumour cells [106]. The proliferation rate of the cancer cells mostly affects chemotherapy potency as cytotoxic agents are effective against rapidly proliferating cells and not on the quiescent ones [107].

The acquisition of chemotherapy resistance is one of the main reasons for the poor survival associated with ovarian cancer, and is almost inevitably a feature of recurring disease [7]. Although the majority of patients respond well to first line of chemotherapy, more that 70% will relapse during the first two years and will eventually develop platinum resistance [8,

108]. Given that there are a number of processes that mediate the cytotoxic effect of cisplatin, ranging from the initial entry of the drug in the cells to the activation of apoptotic pathways, interference in any of these processes will inevitably lead to cisplatin resistance. The complexity of the biology of cisplatin mode of action is indicative of the multifactorial nature of the mechanisms that underpin cisplatin resistance [86].

Several mechanisms of drug resistance have been postulated and have been the subject of a number of clinical trials [7, 77, 94]. The mechanisms underpinning resistance to cisplatin have been classified in four functional categories [77]: i) those that affect the level of intracellular accumulation of cisplatin (pre-target resistance); ii) those related to the genotoxic damage induced by the binding of cisplatin to DNA and its repair (on-target resistance); iii) those affecting the lethal signalling pathways that are activated by DNA damage (post-target resistance) and iv) those involving signalling pathways that deliver compensatory survival signals not directly activated by CDDP (off-target resistance). A summary of these mechanisms is presented in Figure 1-3 [94].

Pre-target resistance

A decrease in the intracellular accumulation of cisplatin is a key mechanism tumour cells have developed to overcome cisplatin induced cytotoxicity [86]. In contrast to the initial assumption for its passive diffusion to the intracellular space [68], it has now been proven that cisplatin is actively pumped in and out of the cells through plasma membrane transporters [109]. Copper transporter receptor 1 (CTR1) binds both copper and cisplatin and is a key mediator of cisplatin intake. Studies on embryonic fibroblasts of CTR1 knockout mice have shown that they accumulate significantly less amount of cisplatin compared to the wild type ones and are resistant to its cytotoxic effects [72, 110]. Cell treatment with copper leads to a decreased cisplatin intake and consequently decreased cytotoxicity [111], whereas

copper chelators are found to increase the cisplatin intake [112]. Apart from a decrease in intake, a reduced accumulation of cisplatin can also be achieved by an increase in its efflux.

A number of other membrane transporters have been found to play a significant role in mediating cisplatin efflux and hence promoting cisplatin resistance. These include the P-type ATPases copper efflux transporters ATP7A and ATP7B, the ATP-binding cassette subfamily C member 2 (ABCC2), also known as MRP2 as well as the copper transporter receptor 2 (CTR2) [113]. Increased expression of CTR2 has been associated with cisplatin resistance in ovarian cancer cell lines, while a positive CTR2/CTR1 ration is a prognostic factor for poor survival outcomes [114]. The ATPase MRP2 has been recognised as another important mediator of cisplatin resistance. This is overexpressed in cisplatin resistant cell lines [115] and has been associated with poor treatment and survival outcomes [116]. Depletion of MRP2 via RNA interference has been found to resensitise hepatocellular and oesophageal cancer cells to cisplatin [116, 117].

Of great importance has been the discovery of a strong association between the expression of the copper efflux ATPases ATP7A and particularly ATP7B with cisplatin resistance [118, 119]. The fundamental biological function of these proteins is to maintain copper homeostasis and their deregulation has been previously associated with Menkes (ATP7A) and Wilson's diseases (ATP7B) [120, 121]. Apart from copper, cisplatin has also been identified as a substrate of both transporters, and was found to compete with copper for its transport [122]. ATP7A regulates the accumulation of cisplatin via sequestering it intracellularly, whereas ATP7B mediates its efflux [123]. Both proteins are highly expressed in a variety of cisplatin resistant tumours [110] , however the association of ATP7B with cisplatin resistance has been more robust. In particular, while ATP7A overexpression produced cisplatin resistance in the ovarian cancer cell line OV2008 [119], its depletion via RNA interference in the resistant ovarian cancer cell CP-20 did not resensitise the cells to

platinum [124]. Furthermore, in the latter study, there was no statistically significant difference in the level of expression of ATP7A between the sensitive (A2780) and resistant (CP-20) cells, which was noted for ATP7B [124]. ATP7B has been found to be overexpressed in several human solid tumours including prostate, ovarian, gastric, breast, non-small cell lung cancer and oral squamous cell cancer [114, 125-128] . Overexpression is directly linked with development of cisplatin resistance, and this has been consistently demonstrated in a number of ovarian cancer cell lines [126, 129].

Another mechanism of pretarget cisplatin resistance is its increased inactivation through binding to cytoplasmic thiol-containing "scavengers" such as glutathione (GSH) and metallothioneins [74]. GSH levels are elevated in cisplatin resistant cell lines [130] and in a panel of ovarian cancer cell lines its levels have been directly correlated to the development of cisplatin resistance [131]. Similarly, studies on human and murine models have shown that increased levels of metallothioneins have been associated with cisplatin resistance [132].

On-target resistance

Enhanced DNA damage repair also plays a key role in the development of platinum resistance and is mediated predominantly via the Nucleotide Excision Repair (NER) and to a lesser extent via the Mismatch Repair (MMR) system, although some of its components are involved in the process [83, 85]. The NER system comprises more that 20 proteins, including the excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1) endonuclease [133, 134]. This incises bulky DNA-cisplatin adducts and its expression has been associated with poor survival outcomes and predicted cisplatin resistance in several human cancers [135-140]. It is of note that ERCC1 is not a reliable biomarker of NER proficiency as initially thought [141]. The MMR system includes proteins such as mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1), and is also involved in detecting damaged DNA. However, in cases of extensive damage, the MMR eventually fails to repair it and

results in the induction of pro-apoptotic signals [142]. In line with this, MLH1 and MSH2 genes are often mutated or downregulated in cases of acquired CDDP resistance [143, 144]. However, overexpression of MSH2 in non-small cell lung cancer patients yielded more favourable survival outcomes, when compared to those who did not receive adjuvant chemotherapy [145]. This discrepancy may indicate the binary role of an effective DNA repair system, which in cases untreated tumours can be protective against relapse, but in the setting of chemotherapy treatment it results in the development of resistance. The MMR system is further involved in cisplatin resistance as defects in its components MLH1 and MSH6 have been associated with increased translesion synthesis (TLS) [142], a process that allows DNA to continue replicating outside the cisplatin-DNA adducts [146].

Cisplatin can induce double-strand breaks which are repaired by the homologous recombination machinery (HR) [147], key components of which are encoded by the breast cancer 1 early onset (BRCA1) and breast cancer 2 early onset (BRCA2) genes [148, 149]. Therefore, BRCA1 and BRAC2 mutations result in HR deficient tumours which are more susceptible to cisplatin treatment [150-152].

Apart from binding to nuclear DNA, cisplatin also exerts its cytotoxic action also via its cytoplasmic targets. Only a small number of these interactors have been identified, and includes proteins such as HSP90, myosin IIa, ribosomal protein L5 and the voltage – dependent anion channel 1 protein (VDAC1) as well as the mitochondrial genome [81, 153]. It is of note that depletion of VDAC1 confers resistance to cisplatin [97], whereas overexpression has been found to increase tumour's chemosensitivity [99].

Post-target resistance

Post-target resistance involves alterations and defects in the complex mechanisms that are responsible for the detection of cisplatin induced damage and the orchestration of lethal apoptotic signals leading to cell death [86, 94]. Cells tend to activate an adaptive response

aimed to maintain cellular homeostasis and involve anti-apoptotic signal transduction [154]. When the re-establishment of homeostasis is not achievable, lethal signals are activated resulting to apoptosis [155]. In the case of cisplatin treatment, these involve the (usually p53 mediated) activation of BAX and BAK1 proteins as well as the accumulation of reactive oxygen species (ROS), followed by the opening of the permeability transition pore complex (PTPC) [88, 156]. These processes result in mitochondrial outer membrane permeabilisation (MOMP), and consequently in the activation of caspase dependent and independent cell death mechanisms [157]. Resistant tumours have developed mechanisms to interfere with this apoptotic process and escape death. A main mechanism is the inactivation of TP53, which disrupts the activation of apoptotic signals and consequently makes the tumour cells tolerant to the DNA damage [158]. The association between TP53 deficiency and cisplatin resistance has been demonstrated *in vitro* as well as in the clinical setting [158, 159]. Cell lines harbouring TP53 mutation are less amenable to cisplatin than their wild type counterparts [160, 161]. Similarly, ovarian cancer patients with TP53 deficient tumours do not benefit from cisplatin chemotherapy, when compared to those with wild –type TP53 tumours [162].

Preclinical studies have shown that the pro-apoptotic signal transducers MAPK1 (also known as p38 ^{MAPK}) and c-Jun N terminal kinase 1 (JNK1) are inactivated in cisplatin resistant cells, preventing the subsequent activation of the pro-apoptotic FAL/FASL system [163, 164]. However, no direct correlation between the levels of MAPK proteins and cisplatin sensitivity has been yet established in the clinical setting.

Changes in the expression levels of factors directly involved in apoptosis (such as BCL2 family members, caspases, death receptors and Inhibitor of apoptosis (IAP) family members) also play a major role in promoting chemoresistance [88, 165, 166]. The association of the anti-apoptotic proteins BCL2 and survivin with cisplatin resistance has been demonstrated in clinical studies [167-171]. Indicative of their significance is the development of a small

molecule inhibitors against each of these factors which have been investigated in the early phase clinical trials. [172-174].

Off-target resistance

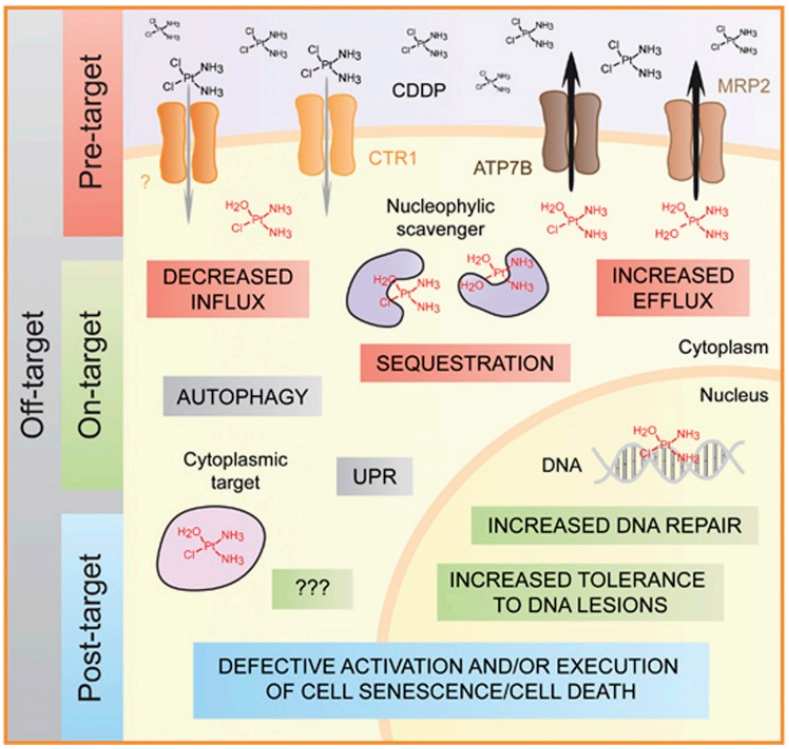
Cancer cells can evade the cytotoxic effects of cisplatin by alterations in molecular circuits which promote cell survival and are not directly activated by cisplatin [94].

The overexpression of the ERBB2 (HER-2) oncogene, which is seen in several types of cancer including ovarian [175], has been associated with cisplatin resistance, exerting its pro-survival effects via the regulation of Pi3K and MAPK signalling pathways [176-178] .

The dual-specificity Y-phosphorylation regulated kinase 1B (DYRK1B or MIRK) is a conserved kinase overexpressed in a number of malignancies, which promotes cisplatin resistance by increasing the expression of antioxidant enzymes [179]. Depletion of DYRK1 in lung and ovarian cancer cells, results in the establishment of ROS and consequent resensitisation to cisplatin [180, 181].

Components of the autophagic pathway have also been involved in promoting cisplatin resistance. In particular lung and ovarian cancer cells were found to progressively overexpress proteins involved in autophagy, such as LC3-II and p62/SQSTM1 respectively, during gradual acquisition of cisplatin resistance [182, 183].

Proteins such as the heat shock protein 27 which act as molecular chaperones in a number of processes and mediate the cellular stress response, have been found to be overexpressed in a number of malignancies and have been associated with resistance to cisplatin treatment [184-186].



Pre target mechanisms
Defective uptake
Increased efflux
Drug deactivation

On-target mechanisms
Increase in DNA repair (NER)
Defective MMR
Increased translesion synthesis
Increase in DNA damage tolerance
Increased HR proficiency

Post-target mechanisms
Loss of p53 function
Downregulation of pro-apoptotic factors (BAX or BAD)
Upregulation of anti-apoptotic factors (BCL2)
Suppressed caspase activity
Deregulation of MAPK pathway

Off -target mechanisms
HER-2/neu overexpression
Enhanced Pi3K/AKT activity
DYRK1B upregulation
Heat shock protein upregulation

Figure 1-3 Mechanisms of cisplatin resistance

Image adapted with permission from Galluzzi et al 2014 [94]

Overview of the molecular mechanisms underpinning cisplatin resistance in malignant cells. These processes are clustered in four functional categories 1) those preceding CDDP binding and resulting in a decrease in its intracellular accumulation (pre-target resistance; 2) those that promote the ability of cancer cells to repair the CDDP induced DNA damage or increase their tolerance to DNA lesions (on-target resistance) 3) those impairing the recognition of DNA damage and consequent activation of pathways leading to cell senescence or apoptosis (post-target resistance) and 4) those not directly elicited by cisplatin, but opposing to CDDP toxicity by stimulating pro-apoptotic signals.

The multifactorial nature of cisplatin resistance is evident as the cell employs a combination of non-overlapping mechanisms to evade its cytotoxic effects. This partly explains the insufficiency of current treatment strategies to desensitise malignant cells to cisplatin agents [94].

(CDDP: cisplatin; ATP7B: copper efflux ATPase; CTR1: copper influx transporter; MRP2; multidrug resistance associated protein 2; aquated cisplatin is depicted in red colour).

1.3.3 SUMMARY

Cisplatin is a key chemotherapeutic compound and the backbone of treatment for a wide range of malignancies over the last four decades. Despite its sustained efficacy in cases of germ cell malignancies, the majority of other tumours will eventually develop resistance. Cisplatin exerts its cytotoxic action predominantly via binding to DNA and causing irreparable damage, which consequently results in the activation of apoptotic pathways and cell death. However, cancer cells have developed a number of mechanisms to evade the cytotoxic effects of cisplatin. These encompass several non-overlapping processes which result in a reduction of intracellular cisplatin accumulation, efficient DNA repair, impairment of the transmission of apoptotic signals and stimulation of pro-survival pathways. It is now evident that cisplatin resistance is multifactorial and understanding the complexity of its underpinning mechanisms has been pivotal in order develop new therapeutic approaches.

1.4 POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION IN CANCER

1.4.1 RNA BINDING PROTEINS

Post-transcriptional regulation encompasses all the events that RNA transcripts undergo which determine their fate. For mRNAs, this ranges from their capping in the nucleus, to their translation or degradation in the cytoplasm. The role of post-transcriptional regulation in tumourigenesis and cancer progression has now become more evident as it provides an effective and rapid way for the tumour cells to adjust their gene expression [187-189]. A number of key processes involved in cancer initiation and progression such as cell proliferation, differentiation, invasion, metastasis, apoptosis and angiogenesis are controlled by post-transcriptional mechanisms [188, 190-192]. Further, cancer cells have also been found to employ such mechanisms in order to overcome genotoxic stress [193]. RNA binding proteins (RBPs) are key post-transcriptional regulatory factors, as they interact with the RNA and are involved in all aspects of RNA processing [194].

Nascent mRNAs undergo a number of processes before their final translation or degradation which include splicing, capping, polyadenylation, nucleocytoplasmic transport and in some cases subcellular localisation [195, 196]. RNA processing is a complex and tightly regulated series of events controlled by a diverse group of RBPs [197]. RBPs carry a variety of RNA binding domains (RBDs), among which are the RNA recognition motif (RRM), K-homology domain (KH), PAZ and PIWI domains, which mediate their binding to specific sequences or secondary structures of their mRNA targets [189, 198]. Each RBP can carry different combinations of RBDs, which allows a diverse pattern of binding to a variety of RNA targets [198]. Multiple RBPs bind to mRNA transcripts forming dynamic units called messenger ribonucleoprotein complexes (mRNPs), which dynamically change during each step of mRNA biogenesis and regulate its fate in a time and space dependent manner [194].

Emerging techniques such as Cross Linking-Immunoprecipitation (CLIP) and Photoactivatable-ribonucleoside–enhanced CLIP (PAR-CLIP), followed by nucleic acid sequencing have majorly contributed to the recognition of new RBPs in human cell, as well as new RBP binding sites [199-201]. More than 1500 RBPs have been identified in the human genome with the list expected to increase in number and complexity [199, 202].

A study investigating the expression of RBPs in 16 healthy human tissues showed that their level of expression outweighed that of non-RBPs across all examined tissues. Furthermore, RBPs exhibited particularly high levels in gonadal tissues and lymph nodes, indicating the need for extensive of post-transcriptional control in highly dividing tissues [203]. The deregulated expression of RBPs, or mutations in their binding sites have been linked to a number of diseases such as neurological disorders and cancer, including ovarian cancer [203-205].

1.4.2 RNA BINDING PROTEINS IN CANCER

There is accumulating evidence supporting the pivotal role of RBPs in cancer. In an analysis involving nine human cancers, genes encoding for RBPs were found to be expressed at a higher level than other classes of genes for regulatory factors including miRNAs and transcription factors. Further, a subset of 30 RBPs were found to be strongly upregulated in the cancerous tissues, when compared to healthy tissue, suggesting an oncogenic role for these proteins [203]. As yet, only a few RBPs have been extensively studied and linked to cancer, but this number is set to increase as technological advances allow the discovery of new RBPs and their functional characterisation [201, 206]. Among the RBPs whose role in tumourigenesis has been well studied, are the eukaryotic initiation factor 4E (eIF4E), and the proteins La, Sam68 and HuR [197]. Overexpression of eIF4E has been reported in a number of cancers including breast, prostate, gastric, colon, lung, and skin cancers and has been associated with increased translation of a subset of mRNAs encoding proto-oncogenic

proteins [207, 208]. The Sam68 protein has been found to be highly expressed in breast, renal, prostate and cervical cancers and promotes tumorigenesis via the regulation of alternative splicing of cancer related mRNAs such as cyclin D1 [209-211]. Furthermore, along with other splicing regulators, Sam68 has been involved in the cellular response of tumour cells following genotoxic stress , suggesting that RBPs can also play a key roles in promoting resistance to chemotherapy [212]. Apart from changes in mRNA splicing, alterations in mRNA stability have also been involved in tumorigenesis. The HuR /ELAV1 protein is an RBP which is upregulated in several cancers and exerts its oncogenic actions via regulating the stability of mRNA transcripts [192, 213]. The La protein is overexpressed in chronic myeloid leukaemia, cervical cancer, oral squamous cell carcinoma and lung cancer and has been found to regulate the translation of mRNAs involved in cell proliferation, angiogenesis and apoptosis [214, 215]. It has also been associated with cisplatin resistance in head and neck squamous cell carcinoma cells by stimulating the translation of anti-apoptotic factor BCL2 [216]. The key role of post-transcriptional regulation and particularly of RBPs in promoting tumorigenesis and chemotherapy resistance has brought them into focus as potential therapeutic targets opening new therapeutic approaches [217].

1.4.3 EUKARYOTIC MRNA PROCESSING

An understanding of the steps involved in eukaryotic mRNA biogenesis and processing is necessary in order to better appreciate the role of RNA binding proteins. The mRNA life cycle is a rather complex process and several aspects of its post-transcriptional regulation are yet to be fully elucidated. Following transcription initiation, the nascent mRNAs undergo three major processes which occur in the nucleus and include: 5'capping, 3' cleavage and polyadenylation and splicing. Following these steps, functional mRNAs are exported to the

cytoplasm where they are either stored, translated or degraded [196]. All these processes are regulated by complexes of RNA binding proteins [198, 218].

1.4.3.1 Transcription initiation and 5' capping

Eukaryotic mRNA transcription starts with the assembly of the transcription pre-initiation complex (PIC), comprising of RNA polymerase II and several transcription factors, upstream of a gene on the gene promoter. The formation of the PIC depends on the recognition of core promoter elements (CPE), such as the TATA box, by CPE- binding proteins such as TATA binding protein (TBP). Once the PIC is assembled, and after promoter melting and escape have occurred, transcriptional elongation follows until the RNA polymerase reaches the termination site. Multiple transcription cycles can occur on any given gene. [219-221].

Pre-mRNA processing occurs co-transcriptionally and is facilitated by the C-terminal domain (CTD) of the RNA Polymerase II which recruits various co-factors according to the phase of transcription. The CTD gets phosphorylated on the 2nd, 5th and 7th serine residues indicating the different stages of transcription. Once the nascent RNA reaches a length of 25-30 nucleotides, a 7-methylguanosine cap is attached to their 5' end by mRNA capping enzymes [222]. This structure is required for the nuclear export of the mRNA transcripts, as well for cap-dependent translation [223].

1.4.3.2 Elongation, splicing and editing

During transcription elongation, the RNA polymerase does not always proceed at a steady rate but it may periodically pause at specific sequences before recommencing again. More frequently it can stall during the early phase of elongation close to the promoter. This mechanism called promoter- proximal pausing, acts to regulate the transcription rates of

genes with high expression rates and is governed by Negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) [224, 225].

Eukaryotic pre-mRNAs encode protein coding regions (exons) scattered in the genomic DNA among non-coding sequences (introns), which are 10 times lengthier [226, 227]. Each human gene contains an average of eight introns and, as part of mRNA processing, these are removed by mRNA splicing in order to produce mature mRNA, the template for translation by the ribosomes [228, 229]. Alternative splicing involves the skipping and joining of exons in combinations laid out in their genomic order, or inclusion of different exons in each variant mRNA species, allowing for individual genes to encode for multiple proteins with various functions [230]. It has been reported that 92-94% of human transcripts undergo alternative splicing, which in-turn helps explain the diversity of the human proteome and can have further implications for disease and health [231, 232]. Splicing is catalysed by the spliceosome, a large dynamic RNP complex comprising small nuclear RNAs (snRNAs) and more than 150 proteins [194, 233]. Recruitment of the splicing machinery is mediated via phosphorylation of CTD at Sr2 [234]. Despite the fact that splicing can occur post-transcriptionally in the nucleus [235], it has been shown that it can take place co-transcriptionally [236] .

RNA editing occurs co-and post-transcriptionally and involves the modification of primary RNA sequences [237]. It is a rather frequent process with more than 16,905 editing sites identified in mRNA [238]. The main form of RNA editing in mammals is the deamination of adenosine to inosine (A to I editing), a process catalysed by a family of enzymes named adenosine deaminases acting on RNA (ADAR) [239]. Apart from editing sequences, RNA editing can also occur in miRNAs resulting in a change in their targeting specificity [240]. Furthermore, RNA editing can also promote alternative splicing through the deletion or creation of new splicing sites [237].

1.4.3.3 Termination, 3'–end cleavage and polyadenylation

Transcription termination in mammals can occur at any site within few base pairs to several kilobases downstream of the 3'-end of mature mRNAs [241] and is closely coupled to the 3'end processing of the pre-mRNAs [242]. The termination process is complex and requires several proteins which are recruited by the CTD and include the cleavage and polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CstF) and poly(A) polymerase [243, 244]. Eukaryotic protein coding mRNAs carry a conserved hexanucleotide motif AAUAAA at their 3' end, which is followed by a G/U rich sequence. Cleavage and polyadenylation occurs in between these sequences at approximately 10-30 nucleotides downstream of the AAUAAA sequence, which is recognised by the cleavage and polyadenylation specificity factor (CPSF) [245]. Following transcription of the poly(A) signal by the RNA Polymerase II, the process pauses and the pre-mRNA undergoes endoribonucleolytic cleavage, which is followed by polyadenylation of the upstream product while the downstream product gets degraded [221].

1.4.3.4 Nuclear export of mRNA transcripts

To facilitate their nucleocytoplasmic export, mature mRNAs assemble into mRNP complexes with a large number of RNA binding proteins which include poly A binding protein (PABP), heterogeneous nuclear ribonucleoproteins (hnRNPs), cap-binding proteins CBP20 and CBP80, as well as splicing factors [246]. These mRNPs are exported from the nucleus via large protein complexes, named nuclear pore complexes (NPC), that cross the nuclear envelope [247] . The mRNA export in metazoans is tightly coupled with splicing, and it is the splicing machinery that recruits the transcription–export complex (TREX) into nascent mRNAs. The nuclear RNA export factor 1 (NFX1) interacts with the mature mRNAs via TREX components and splicing factors, and links the mRNP with the NPC to expedite its export [248]. Once the mRNPs pass through the nuclear membrane, they undergo

remodelling which involves removal of the proteins bound to it by the helicase Dbp5 (or DDX19 in humans) [249].

1.4.3.5 Cytoplasmic localisation of mRNA transcripts

Following nuclear export, the localisation of the mRNA transcripts in the cytoplasm is a tightly regulated process which governs the temporal and spatial expression of a given protein [250]. This process also ensures that according to their function, specific proteins are located in the relevant cellular compartments. For example, transcripts encoding mitochondrial proteins are localised in the mitochondria [251]. A typical example of targeted mRNA localisation is the accumulation of b-actinin transcript at the leading edge of migrating cells [252]. Aberrations in the localisation of specific transcripts can lead to disease and is indicative of the importance for this process to be highly regulated. A characteristic example is that of myelin basic protein (MBP) mRNA, which is normally localised to myelinating oligodendrocyte processes and where a disruption in its localisation results in ectopic myelination in vivo [253]. The cytoplasmic localisation of mRNA transcripts is governed by several mechanisms, which include nuclear events, the presence of cis-acting localisation elements as well as extracellular pathways [250]. Key mediators of mRNA localisation are RNA-binding proteins which act on sequences in the 3'UTR of transcripts, where they either transport them actively or affect their stability in a spatial manner [254]. For example, the RNA binding protein Staufen interacts with the 3' UTR of bicoid transcripts and directs their localisation in the Drosophila embryos [255].

1.5 THE LARP PROTEIN FAMILY- AN OVERVIEW

The La-Related Protein (LARP) family comprises a group of highly conserved proteins, all containing a "La domain" consisting of a 90-aminoacid "La motif" (LAM) and an "RNA recognition motif" (RRM) separated by a short linker [256, 257]. LARPs have RNA-binding properties and are widely found in eukaryotes [256]. Evolutionary and structural analysis of these proteins has led to their classification into five subfamilies which are present in humans and include: Genuine La (known as SS-B and recently named LARP3) which was the first member to be described; LARP1 (variants 1a and 1b); LARP4 (variants LARP4a and 4b), LARP6 and LARP7 [256, 258]. The structural characteristics of the LARPs have been described by Bousquet-Antonelli & Deragon [256] and Bayfield et al.[257] and are summarised in Figure 1-4.

The exact position of the "La domain" differs among the LARP members; in genuine La it is located proximally to the N-terminus, whereas in the other LARPs, it has a more central location. Genuine La and LARP7 carry a classical RRM motif with all other LARPs sharing a non-typical "RRM-like" (RRM-L) motif. These two motifs have co-evolved and are both necessary for the RNA-binding function of the LARPs [256, 259]. Genuine La and LARP7 have an additional C-terminal RRM, named RRM2. The LARP1 subfamily members possess a highly conserved unique C-terminal region comprised of triplicate amino acid repeats. This was initially named the "DM15/LARP1" motif but it is now also known as the LARP1 motif due to the fact that it is uniquely found in LARP1 [256]. It has been recently found that the LARP1 motif binds directly to 5-TOP sequences [260]. LARP6 carries a "SUZ-C" motif of unknown function at its extreme C-terminus. This motif has been also identified in the C-termini of other RNA-binding proteins and is speculated to be involved in their subcellular localisation [256]. Contrary to the other family members, LARP4a and b do not carry any additional C-terminal motifs but have an atypical N-terminal PAM2 domain (the PAM2w)

which binds polyadenylate binding protein (PABP) [261].

Despite sharing similar RNA-binding domains, the LARP subfamilies do not exhibit functional homogeneity and appear to have markedly different cellular functions [256, 258]. They are involved in several aspects of post-transcriptional regulation and/or translation and there is increasing evidence associating each of these proteins with human disease and, in particular, with cancer [258].

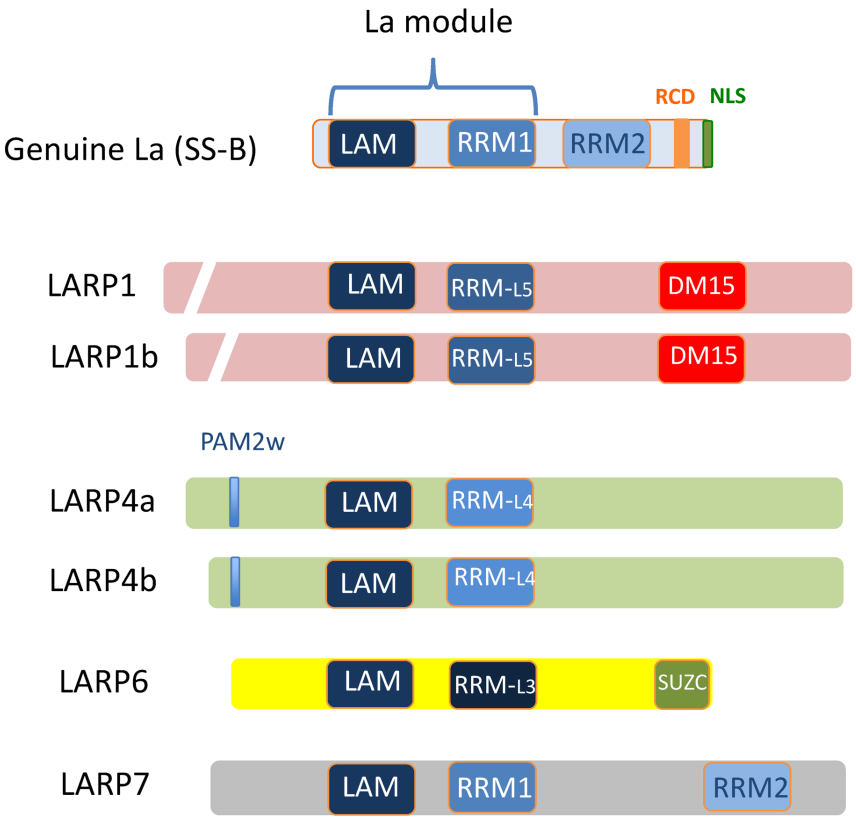


Figure 1-4 Overview of the principal domains present in the members of LARP family.

Abbreviations: DM15: DM15-repeat containing region ("DM15 region") also known as "LARP1 motif"; LAM: La Motif; NLS: Nuclear localisation signal; PAM2w: Atypical PAM2 domain; RCD: RNA chaperone domain; RRM: RNA Recognition Motif; RRM-L: RNA recognition-like motif; SUZ-C: SUZ-C domain.
Originally adapted from Bayfield et al [257] and reproduced with permission from Stavraka et al [258].

1.5.1 GENUINE LA/LARP3/LA/SSB

Protein La is the smallest member of the LARP1 superfamily (46kDa) and the most abundant one [262]. It has been studied to a much greater extent than the other members of the LARP family, as it was first identified as an autoantigen of autoimmune disorders and especially Sjogren's syndrome [263, 264]. It plays a fundamental role in cell biology and is required for the embryonic development of mice and Drosophila [265, 266]. Protein La is mostly cytoplasmic but has the ability to shuttle between the nucleus and the cytoplasm [267]. It is a multifunctional protein involved in a number of cellular processes which include cellular and viral RNA metabolism such as the processing of RNA polymerase III transcripts, microRNA (miRNA) processing and mRNA and t-RNA stabilisation [262, 268-270]. LARP3 has a chaperone activity and has been found to fold around the 3'UUU_{OH} termination motifs of misfolded, nascent RNA polymerase III transcripts via its La domain, ensuring correct folding and protection from exonuclease digestion [262]. Such transcripts include pre-5S rRNAs [271], and also small RNAs such as the U3 snoRNA in yeast [272]. LARP3 also acts as a chaperone to non-3'UUU_{OH} bearing targets, contributing to the stabilisation of pre-tRNAs by protecting their 3'end [257, 262, 273]. This chaperone activity seems to be also conserved in LARPs 4, 6 and 7 [274]. Protein La has also been found to associate with nascent pre-miRNAs and protect them from nuclease digestion. The majority of these transcripts do not carry poly-UUU tails. However, they are recognised by protein La through their characteristic stem loop conformation. It is of note that while the La domain is required for the 3'UUU_{OH} and pre-tRNA binding, the entire LA-RRM1-RRM2 stretch is needed for the non-3'UUU_{OH} mediated interactions [275].

Apart from the role LARP3 holds in post-transcriptional processes, it has also been involved in mRNA translation. It has been found to play a pivotal role as an IRES *trans*-acting factor (ITAF) in promoting RNA translation of several viral targets including poliovirus [276, 277]

Hepatitis C RNA [278-280]. Other IRES-containing mRNA targets of LARP3 include Cyclin D1 [215], XIAP [281], the cell cycle activator Murine Double Minute 2 (MDM2) [282] and the chaperone immunoglobulin heavy chain binding protein BiP [283].

Apart from the recognised role LARP3 holds in promoting IRES translation, it has also been found to regulate the translation of 5-TOP mRNAs. TOP mRNAs are named so due to the fact that they carry a stretch of 4-14 pyrimidine residues followed by a GC-rich region adjacent to their 5'm^7Gpp cap. Such transcripts encode for proteins which are components of the translational apparatus such as ribosomal proteins [284]. The role of LARP3 in the translation of 5-TOP mRNAs is controversial as in Xenopus it was initially found to promote 5-TOP translation [285], whereas in human cells it exerted an inhibitory activity [286]. It has been shown that LARP3 function and subcellular localisation is dictated by its phosphorylation status. In particular, upon phosphorylation by the protein kinase CK2 at serine 366, protein La is found in the nucleus bound to nascent to tRNAs and activating their assembly. Non-phosphorylated LARP3 is located in the cytoplasm where it is bound to 5-TOP mRNAs repressing their translation [287].

1.5.1.1 LARP3 and cancer

There is accumulating evidence associating LARP3 with cancer development. LARP3 expression is upregulated in a number of cancers, such as oral squamous cervical and lung cancers [214, 215, 288] compared to benign controls, and also in several cancer cell lines [289]. The oncogenic role of LARP3 has been linked to its target transcripts, particularly Cyclin D1 and MDM2, whose translation is promoted in an IRES – dependent manner [215, 282]. Both of these proteins have been independently associated with cancer [290, 291]. In hepatocellular cancer, protein La has been linked to epithelial mesenchymal transition (EMT) by promoting the IRES-mediated translation of Laminin B, a known EMT driver [292].

In mouse glial cells, protein La gets phosphorylated by AKT at T301, which triggers its translocation to the cytoplasm where it plays a key role in the regulating the translation of key mRNA encoding cancer genes such as VEGF, PDGFA, and BCL2L11 [293] .

DNA damage induced- apoptosis results in the proteolytic cleavage of LARP3. Its N-terminus localises in the cytoplasm and binds mRNA transcripts promoting IRES-mediated translation [294]. LARP3 is known to promote the translation of the X-linked inhibitor of apoptosis protein (XIAP), which inhibits apoptosis upon DNA damage [281]. This indicates a potential role for LARP3 as an apoptosis inhibitor in response to apoptotic stimuli mediated by genotoxic damage.

Protein La has also been associated with haematological malignancies. In myeloproliferative diseases carrying JAK2 mutation (V617F), protein La enhances the MDM2 translation resulting in p53 degradation and cell proliferation [295]. A similar role for protein La has been identified in Chronic Myeloid Leukaemia (CML) where the protein product of the fusion BCR/ABL oncogene upregulates protein La expression which in turn promotes translation of MDM2 [282].

1.5.2 LARP 4A AND 4B

Two paralogues of LARP4 have been identified thus far: LARP4a and LARP4b (formerly known as LARP5) encoded at 12q13.12 and 10p15.3 respectively [256]. They share 37% overall amino acid identity, with 74% identity of their La domains, and 53% sequence similarity [257]. The La motif of LARP4a and 4b is less conserved compared to other family proteins such LARP3 and LARP7, indicating that they may have different mRNA targets [257]. Both are predominantly cytoplasmic and accumulate in stress granules upon treatment with sodium arsenite [261]. They possess an N-terminal, non-canonical PAM2 domain (PAM2w) which, in conjunction with the La domain, mediates its binding to Poly A binding proteins (PABPs) [261, 296]. LARP4a and LARP4b have roles in stimulating mRNA

translation not only through their interaction with the cytosolic PABP1 but also via binding the scaffold protein RACK1[261, 297] which interacts with the 40S ribosomal subunit [298]. In support of such a role, proteins LARP4 and 4b were found to co-sediment with the 40S ribosome component with their knockdown resulting in 20-40% decrease in protein synthesis [297]. Furthermore, both LARP4a and LARP4b have been found to promote mRNA stability [261, 299]

Contrary to other LARPs, LARP4a protein lacks an additional C-terminal RNA binding motif which could possibly limit its RNA binding capacity compared to the other LARP proteins. The N-terminal region of LARP4 shows greater affinity for poly (A) than poly (U) sequences and does not appear to bind poly (C) or (G). A minimum of 15 nucleotides are required for robust poly (A) binding [261], whereas LARP3 requires a 10-nucleotide sequence with a minimum of two terminal uridylate residues to achieve high affinity binding [300]. The LARP4a interactome is yet to be fully characterised, however a LARP4 RIP CHIP analysis has identified approximately 2000 mRNA targets in complex with the protein with no significant enrichment for functional (GO) terms or pathways [261]. The mRNA interactome of Protein LARP4b was explored with the use of Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP). This showed that LARP4b interacts with a large number of cellular mRNAs binding an AU rich element in their 3' UTRs [299].

1.5.2.1 LARP4a/ 4b and cancer

There is limited amount of evidence linking LARP4a and LARP4b to cancer. Unlike other LARPs, the expression of LARP4 is negatively associated with cancer, and LARP4a knockdown was found to promote cell migration in a prostate cancer cell line (PC3) [301]. Similarly, protein LARP4b was identified as a tumour suppressor in glioma cell lines with its overexpression leading to mitotic arrest and apoptosis [302].

1.5.3 LARP6

Protein LARP6, originally known as Acheron, was first identified in the moth species and associated with programmed cell death at the end of metamorphosis [301]. In mammals, it is highly expressed in neuronal, skeletal, cardiac and testicular tissues [303]. Apart from the N-terminal La domain, LARP6 possesses a C-terminal conserved SUZ-C motif which holds mRNA binding capacities [304]. LARP6 carries a nuclear localisation signal (NLS) as well as functional nuclear export sequence (NES) and is located in both the nucleus and cytoplasm [303, 305].

The predominant role of protein LARP6 in benign cells is the regulation of collagen I synthesis. Collagen alpha 1(I), alpha 1(II) and alpha 1(III) mRNAs are directly bound to LARP6 via the conserved stem-loops found in their 5' UTR, and this interaction regulates their subcellular localisation [306]. LARP6 recognises and binds the collagen alpha mRNA transcripts via its "bipartite" RNA domain, which comprised of its La motif and an adjacent N-terminal 40-amino acid sequence only present in LARP6 [256, 306]. Cai et al demonstrated that LARP6 overexpression inhibited translation of all three collagen alpha mRNA transcripts and the same effect was also noted following LARP1 knockdown [306]. Apart from collagen alpha, LARP6 also binds vimentin intermediate filaments, non-muscle myosin, RNA helicase A and protein STRAP, all of which sustain the production of collagen alpha 2, facilitating the generation of heterodimeric collage I fibres during the process of reparative or reactive fibrosis [307, 308].

LARP6 appears to play an important role in myogenesis [309, 310]. It has been found to be activated downstream of insulin-like growth factor (IGF-1) via the Pi3K/AKT pathway signaling in aortic smooth muscle cells [307] and upstream of the MyoD transcription factor, which plays a key role in muscle development [303]. In endothelial cells two isoforms of

LARP6 were identified upon exposure to hypoxia; the shorter isoform 1 was down-regulated while the full-length isoform 2 was not [311].

1.5.3.1 LARP6 and cancer

LARP6 is overexpressed in basal-like invasive ductal carcinomas of the breast compared to normal mammary epithelium. Overexpression in the MDA-MB-231 breast cancer cell line was seen to promote their proliferation and invasion, and was associated with the upregulation of the matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) [305]. In human tumour xenografts, LARP6 was found to act as an oncogene promoting not only tumour growth but also angiogenesis through the upregulation of VEGF expression [305]. The role of LARP6 in angiogenesis was further demonstrated in human umbilical vein endothelial cells (HUVECs), where it was also found to promote proliferation [312]

1.5.4 LARP7

Protein LARP7 is the only family member with a domain structure similar to that of Genuine La, containing a canonical RRM1 domain together with a second C-terminal RRM domain [256, 257]. LARP7 binds RNA polymerase III transcripts and in particular the 7SK snRNA on a 3'UUU_{OH} - dependent manner [313, 314]. Human 7SK RNA is an abundant small nuclear RNA (sn RNA) that, as a part of the 7SK snRNP (small nuclear ribonucleoprotein particle), has an inhibitory effect on the positive transcription elongation factor b (P-TEFb). As a component of the 7SK snRNP, LARP7 indirectly suppresses mRNA transcription [315]. To exert this inhibitory effect both the N- and C-terminal regions of LARP7 are required [316]. The 7SK snRNP has also been found to play an important role in regulating alternative splicing [317].

1.5.4.1 LARP7 and cancer

LARP7 has an inhibitory action on transcriptional elongation through the 7SK snRNP, which suggests a potential onco-suppressive role. Upon LARP7 knockdown, 7SK RNA is degraded, resulting in the release and subsequent activation of P-TEFb protein [316]. The bromodomain protein Brd4 then recruits P-TEFb to chromatin leading to transcription promotion and cell cycle progression [318].

LARP7 expression is downregulated in invasive breast cancer cell lines and its expression is positively correlated with survival outcomes [319]. Knockdown of LARP7 in benign mammary epithelial cells (MCF10A) has been shown to lead to epithelial mesenchymal transition [316]. Furthermore, silencing of LARP7 with short hairpin RNAs in noninvasive breast cancer cell lines promoted malignant progression and metastasis [319]. The role of LARP7 as an oncosupressor was further reinforced by a study of Cheng et al, who showed that its expression is significantly reduces in gastric cancers compared to normal tissue. This downregulation in LARP7 expression occurs early during gastric tumorigenesis. Similarly to what has been observed in mammary epithelial cell lines, LARP7 knockdown increased cell proliferation and enhanced migration in non-cancerous gastric cells [320].

1.5.5 LARP1 AND 1B

Two paralogues of LARP1 have been identified in humans, LARP1 or LARP1a and LARP1b or LARP2, whereas a third gene LARP1c has been described in Arabidopsis [256]. The LARP1 gene is located at chromosome 5q34, while that for LARP1b at 4q28, with these encoding 1096 and 914 amino acid proteins respectively, and sharing 60% sequence identity and 73% similarity [257]. LARP1 is found both in the nucleus and the cytoplasm but is predominantly cytoplasmic. LARP1 proteins possess a LAM/RRM domain at the N-terminus as well as a highly-conserved C-terminal DM15 motif. The latter is found exclusively in the LARP1 proteins and is composed of one to four repetitions of the 40-amino acids-long

domain referenced at the Conserved Domain Database as the DM15 box (smart00684) [256, 321]. It is also known as the "LARP1 motif". To avoid confusion with the La domain, in this thesis, I will refer to this as the DM15 motif. The DM15 motif has been recently found to have an RNA-binding function and to interact directly with 5TOP mRNAs [260]. The majority of publications to date refer to LARP1, than to LARP1b, and this will also be the focus of this thesis in the following sections.

1.5.5.1 LARP1 and RNA binding

LARP1 protein has two signature domains, the LAM/RRM at the N-terminus and the DM15 at the C-terminus, both holding RNA binding properties. Studies involving protein LARP3, have shown high affinity between the LAM/RMM domain and the UUU-OH at the 3' terminus of RNAs [257, 259]. LARP1 has been found to bind mRNA transcripts in two high-throughput studies that used UV-crosslinking to identify the mRNA-bound proteome in human cell lines [199, 322]. Nykamp et al studied LARP1 in C. Elegans and tested whether this binds RNA in vitro using an RNA homopolymere-binding assay. Full length LARP1 was precipitated with both poly (U) and poly (G), but not by poly (A) or poly (C). Fragments of LARP1, each containing the La domain or the DM15 domain, were also assayed and both retained RNA binding capacities but lacked full activity. This suggested that the two domains act co-operatively to achieve optimal RNA binding [321]. Contrary to its C. Elegans orthologue, human LARP1 was found to only interact with poly(A) RNA sequences. Aoki et al used an artificial mRNA transcript containing a part of the human b-actin (ACTB) 3'UTR sequence, which was capped and polyadenylated, to pull down interacting proteins which were further identified by tandem mass spectrometry. LARP1, 1B, 3 and 4 were found among the proteins interacting with the 3' poly(A) tail. The interaction between LARP1 and the poly(A) tail was further investigated with the use of a variety of bait RNAs. It was shown that this interaction was not dependent on the presence of a 5'cap but on the length of the

poly (A) tail and required at least nine adenosine residues. It is of note that the addition of other single nucleotides (guanosine, uridine, cytidine) to the poly (A) tail abolished the interaction.

Apart from interacting with the 3' terminus poly (A) tail, LARP1 has been found to associate with the mRNA 5'cap in an mTOR dependent manner [323]. Tcherkerzian et al [323] used G-sepharose beads coupled to 7-mehtylguanosine (m^7GTP), which mimics the mRNA 5' cap structure, to pull down proteins that interact with the 5' mRNA cap in HEK293 cells. Following high-resolution mass spectrometry, LARP1 was among the precipitated proteins, alongside PABP1 and other translation initiation factors. However, the association between LARP1 and the 5'mRNA cap was considered to be mediated by PABP1, as it was lost after the disruption of the LARP1-PABP1 interaction. Notably, this study identified that LARP1–PABP1 interaction was mediated by the C-terminal region of LARP1, and particularly the DM15 motif.

A number of studies have demonstrated that LARP1 interacts with the 5' terminal oligopyrimidine tract (5'TOP) mRNAs and plays a key role in regulating their translation [323-325]. This association was identified through RNA immunoprecipitation experiments using both endogenous and exogenous LARP1 as a bait [323-325]. Furthermore, Fonseca et al [325] showed that LARP1 directly binds directly the 5'TOP motif competing with the eukaryotic initiation factor eIF4G. This interaction was further confirmed in a different study in which Berman et al showed that that the highly conserved LARP1-specific C-terminal DM15 region of human LARP1 directly binds a 5′TOP sequence [260]. In their latest study, Berman at al. [326] identified that LARP1 binds directly the [m^7G] cap, as well as the adjacent 5'TOP motif of TOP mRNAs. This interaction blocks the eIF4E access to the cap and consequently prevents the eIF4F complex assembly on TOP mRNAs, thereby inhibiting their translation.

In the study by Mura *et al* using the HeLa cell line, LARP1 was found to be complexed to some 3000 mRNA transcripts, as identified by RNA immunoprecipitation and microarray profiling (RIP-Chip) with anti-LARP1 antibody [327]. LARP1 knockdown exerted a binary effect on its target mRNA transcripts, increasing the abundance of some and decreasing others [328].

1.5.5.2 LARP1 and mRNA stability and decay

There is increasing evidence that LARP1 is involved in key post-transcriptional processes involving stability and decay, as well as the translational efficiency of mRNA transcripts.

Both human and Drosophila LARP1 have been found to interact with PABP1, a protein well known for promoting mRNA stability [323, 324, 329, 330]. However, the LARP1-PABP1 interaction has been extensively studied with controversial results. Tcherkezian et al showed that in that in HEK 293 cells, LARP1 interacted directly with PABP1 through the DM15 motif in its C-terminus. The interaction gets significantly decreased in LARP1 mutants with deletions of 150 or 300 C-terminal amino acid residues, so as to include partial to complete loss of the DM15 tandem repeats [323]. A direct LARP1-PABP1 interaction was also supported by Burrows *et al*, as it was found to be resistant to RNAse A treatment in HeLa cells [330]. On the contrary, Aoki et al demonstrated that the RNA dependent nature of this interaction which was abolished upon RNAse I digestion in HEK 293 cells. It is of note that RNAse A specifically cleaves at the 3'side of pyrimidines (uracil/cytosine) and consequently in this study the polyA tail would be largely undigested and maintaining the protein interaction. In Drosophila, the LARP1-PABP interaction was maintained upon treatment with RNAse A, I and V1 as reported by Blagden *et al* [330].

LARP1 was found to bind the poly(A)-tail of a large number of mRNAs independently from PABP1, and to stabilize the transcripts of 5'TOP mRNAs [324]. LARP1 depletion with RNA interference in HeLa cells resulted in a dramatic decrease in 5'TOP mRNAs, without altering

the abundance of non-TOP mRNA transcripts such as GAPDH. The fact that this decrease was noted only for mature 5-TOP mRNAs, with no alteration in their unspliced pre-mRNAs, indicated that LARP1predominantly affects their stability than their transcription [324].

Further evidence of the role of LARP1 on mRNA stability came from the group of Blagden *et a*l. As mentioned above (section 1.4.5.1), this group has demonstrated that LARP1 is in complex with some 3000 mRNA transcripts including mRNAs for mTOR, BCL2 and BIK [327]. LARP1 was found to stabilise the mTOR mRNA via interaction with the 3'UTR [327]. Further work by Hopkins et al in the OVCAR8 cell line [328], showed that LARP1 has a binary effect on its mRNA target transcripts, stabilising some but also destabilising others. In particular, LARP1 was found to stabilise the mRNA of BCL2 but destabilised BIK transcript through an interaction with their 3'UTRs. Such functions inferred a role for LARP1 in promoting tumorigenesis and cancer survival.

The effect of LARP1 on mRNA stability has also been demonstrated in plants and specifically in *Arabidopsis.* In particular, LARP1a was found to interact with an N-terminal region of the 5' exonuclease XRN4 on an RNA-independent manner, and this interaction was suggested to promote mRNA decay upon heat stress [331].

In ovarian cancer cells, upon treatment with sodium arsenate, LARP1 was found to accumulate in both P-bodies and stress granules, which are sites of mRNA degradation and storage respectively [332]. The presence of LARP1 in these sites is indicative of the differential effect this has on mRNA transcripts and a role in determining the RNA fate.

1.5.5.3 LARP1 and mRNA translation

Global protein synthesis rates play a key role in controlling cell growth and consequently governing cell size and proliferation. Impaired regulation of translation is a crucial component of cancer development and progression [333]. Therefore, emerging cancer therapeutic approaches target components of the protein synthesis apparatus [334]. There is

accumulating evidence suggesting a crucial role for LARP1 in regulating protein synthesis. Transient LARP1 depletion through siRNAs in HeLa cells resulted in a 15% decrease in global protein synthesis [330], whereas stable lentiviral knockdown in HEK239 cells caused a 50% decrease [323, 330]. Apart from its established interaction with protein PABP1 [323-325, 330], which is a key translation initiation factor [335]. LARP1 binds independently to the 3' terminus of poly (A) tail of mRNA transcripts, regulating their stability and also their translational activation as LARP1 knockdown as associated with a decrease in polysome assembly [323, 324].

A number of studies have demonstrated a role for LARP1as a key regulator of 5-TOP mRNA translation. Aoki et al found that LARP1 binds the poly A tail and selectively stabilises 5TOP mRNAs [324]. Tcherkezian et al. [323] showed that LARP1 associates with the 5'cap mRNA structure on an mTOR-dependent manner, and plays a significant role in regulating mRNA translation particularly of 5'TOP mRNAs. LARP1 was found in complex with Raptor, regulating the translation initiation downstream from mTORC1 and mediating cell growth and proliferation through the stimulation of 5'TOP mRNA translation. In a consequent study Fonseca *et al*.[325] confirmed LARP1 as an mTORC1 target and reinforced its fundamental role in regulating 5'TOP mRNA translation, showing that it binds directly to the 5'TOP motif. However, contrary to the findings of Tcherckezian et al, this study concluded that LARP1 competes with eIF4G for TOP mRNA binding, preventing the assembly of eIF4F and consequently repressing the translation of 5'TOP mRNAs. In their recent study, Berman et al performed crystal structure studies as well as well as competition and binding assays and suggested that LAPR1 binds the cap and the adjacent 5' TOP motif and prevent the eIF4F assembly [326]. This supports the findings of Fonseca *et al*, showing that LARP1 is a repressor of 5-TOP mRNA translation. A possible explanation of the discrepancy between

these various studies could be that, similarly to protein La, LARP1 alters function depending on its phosphorylation status and therefore can either stimulate or inhibit translation.

1.5.5.4 LARP1 in development

The pivotal role of protein LARP1 in development has been demonstrated by studies done in various species. LARP1 was first identified in Drosophila and shown to be required for oogenesis, spermatogenesis and embryogenesis [336]. LARP1 mutations are associated with male and female sterility as well as with abnormalities in male meiosis [329]. Studies in mouse embryos revealed high LARP1 expression in the spinal cord and dorsal root ganglia, salivary glands, gastrointestinal tract and developing limb buds [336]. Furthermore in C. Elegans, homozygosity for LARP1 truncating mutations were associated with defective oogenesis [321].

1.5.5.5 LARP1 and cancer

There is increasing evidence to link protein LARP1 to carcinogenesis. Mura et al performed a systematic analysis of the Oncomine dataset [337], demonstrating that LARP1 mRNA is highly expressed across a multiple epithelial cancers [327]. They further validated these findings on cervical cancer surgical samples showing LARP1 overexpression at both mRNA and protein levels in the cancerous samples compared to non-cancerous cervical tissue. Notably, LARP1 protein levels followed a stepwise increase from the pre-invasive stages (CIN 1-3) to the development of invasive cervical cancer. LARP1 protein expression positively correlated with disease progression and worse survival outcomes in both cervical and lung cancer [327] . In accordance with these findings, Hopkins et al found that LARP1 mRNA and protein levels were also found upregulated in serous epithelial ovarian cancer compared to normal ovarian tissue and was associated with worse survival outcomes. A TMA analysis of a large cohort of patients within the SCOTROC IV study, identified LARP1

expression as a strong independent prognostic factor for overall survival (OS) and progression free survival (PFS) in ovarian cancer patients [332]. Further studies in hepatocellular cancer (HCC)–derived cell lines [338] and in colorectal cancer tumour samples [339] demonstrated that LARP1 mRNA and protein levels are highly expressed in comparison to benign cells and tissue respectively. In both studies LARP1 was identified as an independent prognostic factor for overall survival with higher LARP1 expression resulting in increased mortality. Interestingly, LARP1 outperformed the established HCC biomarker Alpha fetoprotein (AFP) on its prognostic ability in hepatocellular cancer [338]. The consistency in the findings for LARP1in all the above studies reveals the key role it is likely to play in carcinogenesis.

The importance of LARP1 in cancer was further supported by studies of its biological role. LARP1 knockdown increases apoptosis in a number of human cancer cell lines including the cervical HeLa and ovarian cancer cell lines OVCAR8 and SKOV3 [327, 332]. LARP1 knockdown was shown to have a variable effect on the cell cycle. It resulted in G0/G1 or G2/M arrest in two studies using the non-malignant immortalised HEK293 cells line [323, 330], whereas it had no effect on the cell cycle of the ovarian cancer derived cell lines OVCAR8 and SKOV3 [332]. The inhibitory effect of LARP1 knockdown on cell proliferation was supported by several studies on a variety of cancer cell lines demonstrating that LARP1 is required for clonogenicity [332, 339]. The pivotal role of LARP1 in cell proliferation has been underlined by the key regulatory effects it has on protein synthesis, promoting the translation of 5-TOP mRNAs [323] but also stabilizing the mTOR mRNA transcripts [327].

LARP1 also promotes cell motility and accumulates at the leading edge of migrating cells most likely being involved in the localised mRNA expression and facilitating the development of lamellipodia [330] .

LARP1 is an identified downstream phospho-target of the Pi3K [340] and mTOR [341] signaling pathways both of which are frequently deregulated in cancer and promote cellular growth and proliferation.

Indicative of the central role LARP1 plays in cancer is the fact that its mRNA interactome, identified in HeLa cells by RIP- Chip and consisting of approximately 3000 mRNAs, was enriched for transcripts associated with cancer-related pathways such as mTOR [327].

1.5.5.6 LARP1 and cisplatin resistance

LARP1 overexpression has been associated with disease progression and poor survival outcomes and in a number of human cancers, whereas its depletion results in increased apoptosis [332, 338, 339]. Response to cisplatin chemotherapy is a main determinant of survival outcomes in ovarian cancer patients, but the majority of tumours evade the apoptotic effects of chemotherapy. Given these observations, Hopkins et al [332] investigated whether LARP1 is involved in promoting cisplatin resistance in ovarian cancer. They investigated the effect of LARP1 knockdown with or without cisplatin in the viability and apoptosis of two cisplatin resistant cell lines (OVCA8 and SKOV3). They concluded that the combination of LARP1 knockdown with cisplatin led to 4-fold increase in apoptosis and a significant decrease in viability compared to drug treatment alone. The same outcome was also noted with the isogenic cell lines PE01 and PEO4 which are derived from the same patient before and after the development of cisplatin resistance.

1.5.6 SUMMARY

The La-related (LARP) protein family consists of Genuine La, LARP1, LARP1b, LARP4, LARP6 and LARP7 proteins. All family members share the highly conserved La motif but show variability in their RNA recognition structures which could justify the heterogeneity in their preferred RNA targets and consequently their cellular functions. LARP1 and LARP3 shuttle between the nucleus and cytoplasm, while LARP6 is also present in both subcellular compartments. LARP4 is predominantly cytoplasmic whilst protein LARP7 is nuclear. All LARP family members have been found to be dysregulated in cancer but they exhibit different functions with LARP1, LARP6 and protein LA acting as oncogenes whereas LAPR4 and LARP7 as tumour suppressors.

LARP1 is the largest family member and holds a unique RNA binding motif, the DM 15 motif in its C terminal region. It is highly expressed in a number of malignancies at both the mRNA and protein level and associated with disease progression and poor survival outcomes. LARP1 expression has been identified as a significant independent prognostic factor in ovarian, hepatocellular and colorectal malignancies. LARP1 regulates key cellular processes involved in proliferation such as protein synthesis and promotes cell survival via the post-transcriptional regulation of pro-survival genes such as BCL2. Furthermore, it has been recently shown that LARP1 overexpression is associated with cisplatin resistance whereas its knockdown can resensitise platinum resistant cells to cisplatin chemotherapy. This suggests that LARP1 could be a potential therapeutic target.

1.6 PROJECT AIMS

Ovarian cancer remains the most lethal of all gynaecological malignancies with cisplatin resistance being the main hurdle to overcome. The genetic heterogeneity of ovarian cancer as well as the complexity of the mechanisms that underpin cisplatin resistance require targeted treatment strategies [7, 8]. This has been the focus of intensive research over the past two decades, but with limited success thus far [342]. Advances in RNA capturing and sequencing techniques have identified an increasing number of RNA binding proteins that play a major role as post-transcriptional regulators of gene expression and are implicated in a number of malignancies [197, 203]. The ability of RNA binding proteins to form complex post-transcriptional networks and modulate gene expression on a timely and flexible manner in order to maintain cell homeostasis have brought them to the forefront of research as promising anticancer targets. Such an example is the eIF4E protein, which controls cellular translation, and its overexpression has been associated with several malignancies and correlated with poor survival outcomes [334, 343]. Inhibitors against eIF4E have been developed and are under evaluation in the context of early phase clinical trials [208, 334].

LARP1 is overexpressed in ovarian, hepatocellular, cervical and non-small cell lung cancer and has been associated with worse survival outcomes [327, 332, 338, 339]. Furthermore, in *vitro* and *in vivo* studies from our laboratory have demonstrated that LARP1 is involved in maintaining cisplatin resistance in ovarian cancer cell lines and LARP1 knockdown seems to have a synergistic effect with cisplatin in increasing cell death [332].

The mechanism via which LARP1 exerts its role in cisplatin resistance is yet to be elucidated. The overall aim of this project is to investigate the role of LARP1 in ovarian cancer cisplatin resistance.

In detail:

1. To investigate the molecular mechanism through which LARP1 is involved in promoting chemotherapy resistance by exploring the LARP1 ribonucleocomplex (including mRNA and proteins) before and after genotoxic stress.
2. To further assess the synergistic effect of LARP1 knockdown and platinum *in vivo* and *in vitro*.

2 CHAPTER 2 - MATERIALS AND METHODS

2.1 CELL CULTURE AND DRUG TREATMENT

OVCAR8, OVCAR3, and SKOV3 cell lines were kindly donated by Dr Manuela Mura. A2780 and CP70 cell lines were kindly donated by Mr John Gallon and Mrs Aline Teixeira Marinho. Cells were maintained in RPMI medium (Gibco) supplemented with 10% fetal calf serum (FCS) (First Link), L-glutamine (Life technologies, Paisley, UK) to a final concentration of 2mM and Penicillin/Streptomycin at a concentration of 100IU/mL and 100mg/mL respectively, at 37°C in 5% CO2 atmosphere. OVCAR3 cells were cultured in RPMI (Gibco) supplemented with 20% FCS and 0.01 mg/ml bovine insulin (Sigma-Aldrich, St. Louis, MO). SKOV3 tetracycline inducible (LARP shRNA/GFP shRNA) clones were generated in the lab and kindly provided by Dr Katrina Sweeney. These were cultured in RPMI (Gibco) supplemented with 10% tetracycline depleted FCS serum (Gibco), 0.1% Blasticidin and 0.03% Puromycin [344].

Cisplatin treatment of the used cell lines was guided by the IC50 values, previously established in our lab, according to their resistance status. In detail, the cisplatin resistant cell lines (OVCAR8, SKOV3, CP70) were exposed to cisplatin (Accord Healthcare, Middlesex, UK) at a concentration of 25μM, whereas the sensitive cell lines (OVCAR3 and A2780) were treated at a concentration of 10μM. Gemcitabine (Hospira, Lemington Spa, UK) paclitaxel (TEVA UK, Castelford, UK) and bleomycin (Hospira, Lamington Spa, UK) were used to treat the cells at the stated concentrations which were previously established in our lab.

2.2 PROTEIN EXTRACTION AND WESTERN BLOTTING

Following washing, cells were incubated with protein lysis buffer (1% NP-40, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, supplemented with protease (Roche, Welwyn Garden City, UK)

and phosphatase (Merck Millipore), inhibitors for 30 minutes at 4ºC. Lysates were centrifuged and protein was quantified with the microBCA protein assay kit (Thermo Scientific, Loughborough, UK). Protein samples were boiled with Laemmli buffer and separated by SDS-PAGE using the Xcell Surelock Mini Electrophoresis system (Invitrogen). Proteins underwent wet transfer to nitrocellulose membranes. Membranes were blocked with 5% w/v dry milk in Tris-Buffered Saline supplemented with 1% Tween (TBST) for 1hour and incubated with primary antibodies according to the manufacturers' instructions Table 2-1. Species specific horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Dako. All washes were performed in Tris-buffered saline (TBS) supplemented with 1% TWEEN. Blots were developed using either the Imobilion HRP substrate (Millipore) or Clean Blot^{TM} IP detection reagent (Thermo Fischer) and luminescence was visualised with X-ray films.

Table 2-1 Primary antibodies used for western blotting

Antibody	Species	Supplier	Dilution
LARP1	Rabbit	SDIX	1:2500
YB1	Rabbit	Abcam	1:1000
PABP1	Rabbit	Abcam	1:1000
PABP4	Rabbit	Novus Biologicals	1:2000
HSP60	Rabbit	Abcam	1:5000
ATP7B	Rabbit	Novus Biologicals	1:200
Puromycin	Mouse	Milipore	1:25000

2.3 PROTEIN IMMUNOPRECIPITATION

Cells were either treated with CDDP at the stated (in results) concentration or left untreated and collected by trypsinisation and re-suspended in Polysome lysis buffer (PLB): 20mM Hepes pH 7.4, 150mM KCl, 5mM MgCl2, 0.5% NP40, 400 U/ml RNase inhibitor (Promega), 1mM DTT, 400µM vanadyl ribonucleoside complexes (VRC; NEB), supplemented with protein and phosphatase inhibitor, to preserve the ribonucleoprotein complex (RNPs). The

quantity of cells collected in treated and untreated samples was equal. Floating cells that might have undergone apoptosis were also collected to avoid a bias towards collecting intrinsically resistant cells.

The RNP lysates were stored at -80˚C overnight to complete the lysis and avoid adventitious binding of RNA and proteins [345]. LARP1 was immunoprecipitated with 12µg of rabbit anti-LARP1 polyclonal antibody (SDIX) with equivalent amounts of rabbit IgG isotype control (Cell Signalling Technology, Hitchin, UK) following the method described by Keene et al [345]. For the reverse immunoprecipitations, the following antibodies were used: 10µg rabbit anti-PAPB1 antibody (Abcam); 10µg rabbit anti-PABP4 antibody (Novus biologicals) and 10µg rabbit anti-YB1 antibody (Abcam) respectively. An equal amount of the rabbit IgG isotype control was used in each case. In brief, Protein A-Sepharose beads were incubated with antibody overnight on a rotator at 4˚C. Beads were washed four times with NT2 buffer (50mM Tris-HCL, 150 mM NaCl, 1mM MgCl2, 0.05% NP-40), before adding 750 µL immunoprecipitation buffer (200U/ml RNase inhibitor, 400µM VRC, 1mM DTT, 30µM, 15mM EDTA in NT2 buffer) and the RNP lysates, in a total volume of 1ml. Samples were incubated for 4 hours on a rotator at 4˚C, before washing 4 times with wash buffer (50mM Tris-HCL, 300mM NaCl, 5mM MgCl2, 0.5% NP-40, 1mM DTT). LDS sample buffer (NuPage) (x2) on equal volumes was used for the elution of samples.

2.4 PROTEIN IMMUNOPRECIPITATION WITH RNA DIGESTION

The protein immunoprecipitation protocol (section 1.3) was performed in the presence and absence of Micrococcal Nuclease in the OVCAR8 cell line. LARP1 was immunoprecipitated with 12 µg anti-LARP1 antibody (Novus Biologicals) with equivalent amounts of rabbit IgG isotype control (Cell Signalling Technology, Hitchin, UK). The RNP lysates were incubated

with Micrococcal Nuclease (Thermo Scientific) (1.5 Units per µL of lysate) supplemented with CaCl2 for 30 minutes at 37°C and then resuspended in NT2 buffer (50mM Tris-HCL, 150 mM NaCl, 1mM MgCl2, 0.05% NP-40), to a final volume of 1 ml, before being added to the antibody coated beads. Samples were incubated for 4 hours on a rotator at 4˚C, before undergoing four washes with wash buffer (50mM Tris-HCL, 300mM NaCl, 5mM MgCl2, 0.5% NP-40, 1mM DTT). Samples were eluted with LDS sample buffer (NuPage) on equal volumes.

2.5 RNA IMMUNOPRECIPITATION (RIP)

The exact process described in section 2.3 for protein immunoprecipitation was followed. However, for the purposes of this experiment samples were not eluted with LDS buffer but the RNA co-immunoprecipitated with the protein was extracted from the beads with Trizol (Life Technologies, Carlsbad, CA, USA) and purified with RNA clean-up and concentration micro kit (Norgen Biotek, Thorold, ON, Canada) as per manufacturer's instructions. For cDNA generation, immunoprecipitated RNA was reverse transcribed with the SensiScript RT Kit (Qiagen) as per manufacturer's instructions. RT-qPCR was performed as described in section 2.6. The fold enrichment for each target was measured by comparing the Ct values of LARP1 immunoprecipitated fraction to the IgG Isotype fraction and normalised with the ΔCt formula.

2.6 RNA ISOLATION/RETROTRANSCRIPTION AND RT-QPCR

RNA from cells treated with siRNAs against LARP1 and YB1 was extracted with the RNeasy plus mini kit (Qiagen) and retrotranscribed using MMLV reverse transcriptase (Promega) following the manufacturer's protocol.

Relative RNA abundance was quantified by Real Time - quantitative Polymerase Chain

Reactions (RT-qPCR). Exon-spanning TaqMan RNA expression assays (Invitrogen- Table 2-2) and Universal Master Mix II (Invitrogen) were used for all RT-qPCR experiments on a 7900HT analyser (Applied Biosystems, Paisley, UK).

The fold enrichment of immunoprecipitated RNA was measured by comparing the Ct values of LARP1-IP or YB1-IP fractions to the IgG-IP fraction and normalised using the ΔΔCt formula. 18S rRNA was used as control reference gene and relative mRNA levels were calculated using the ΔΔCt formula.

Table 2-2 Primers used in RT qPCR

Gene	Taqman assay ID
ATP7A	Hs00163707_m1
ATP7B	Hs01075310_m1
18S rRNA	Hs03003631_g1
28S rRNA	Hs03654441_s1
LARP1	Hs00391726_m1
BCL2	Hs00608023_m1
ABCB1	Hs00184500_m1
SLC30A1	Hs00253602_m1
DDB2	Hs03044949_m1
YB1	Hs00358903_g1

2.7 ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (UHPLC) - TANDEM MASS SPECTROMETRY

Following immunoprecipitation of the LARP1 protein complex in cisplatin treated and untreated OVCAR8 and OVCAR3 cell lysates, the LARP1 protein interactors were identified by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) at the University of Dundee Proteomics Mass Spectrometry Facility. A second biological repeat of the experiment was performed and sent for UHPLC tandem mass spectrometry at the University of Oxford Advanced Proteomics Facility.

The OVCAR8 cell line LARP1-immunoprecipitation proteins obtained following RNAse digestion also analysed by UHPLC tandem mass spectrometry at the University of Dundee.

2.7.1 PEPTIDES ANALYSIS PROTOCOL

Peptides were analysed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) using a Dionex Ultimate 3000 UHPLC (C18 column PepMap RSLC 100 with 2 μm particle size, 100 Å, 75 μm x 50 cm; Thermo) coupled to a Q Exactive HF tandem mass spectrometer (Thermo Scientific, Bremen, Germany). The samples were loaded in a 0.1% Trifluroacetic acid (TFA) and analysed using a 1 hour linear gradient at a flow rate of 250 nl/minute. The gradient used to elute the peptides started at 3 minutes with 2 % buffer B (0.1% FA and 5% DMSO in CH3CN) increasing to 5% buffer B by 6 minutes followed by an increase up to 35% by 63 minutes. The column was washed twice 10 minutes with 99% of buffer B. The total length of the analysis was 100 min to allow column re-equilibration. The data were acquired with a resolution of 60,000 at m/z 200 with a maximum injection time of 100 ms and the lockmass (445.120025 m/z) was enabled. The resolution for the MS2 HCD spectra was set to 15,000 at m/z 200 with a maximum injection time of 41 ms. The precursor ion selection was set to Top 15, the Dynamic Exclusion was 27

seconds and fragmentation performed in Higher-energy C-trap dissociation (HCD) mode with Normalised Collision Energy of 28.

2.7.2 PROTEOMIC DATA INTERPRETATION

The raw data were imported in Progenesis QI for proteomics (v2.0.5556.29015) for data analysis. The FASTA file for the human database used was UPR_HomoSapiens 20141015 (85,889 sequences; 33,866,397 residues).

The mass spectral data were interpreted with Mascot software. The identified proteins were ranked according to their "protein score" which reflects the sum of the scores of all the spectra that could match to amino acid sequences within that protein. The higher the score, the more significant the match is and the more likely it is for a protein to be present in the investigated sample.

Within each biological repeat of the experiment the identified proteins of LARP1 complex were checked against the proteins bound to the negative control (IgG protein). Proteins present in the negative control were excluded from further analysis for "non-specific" binding. The outcomes of the two biological repeats where then pooled together. Only those proteins, which were present in both biological repeats and absent from the negative control samples were considered for further evaluation as potential LARP1 interacting targets. The data analysis process is summarised in Figure 2-1.

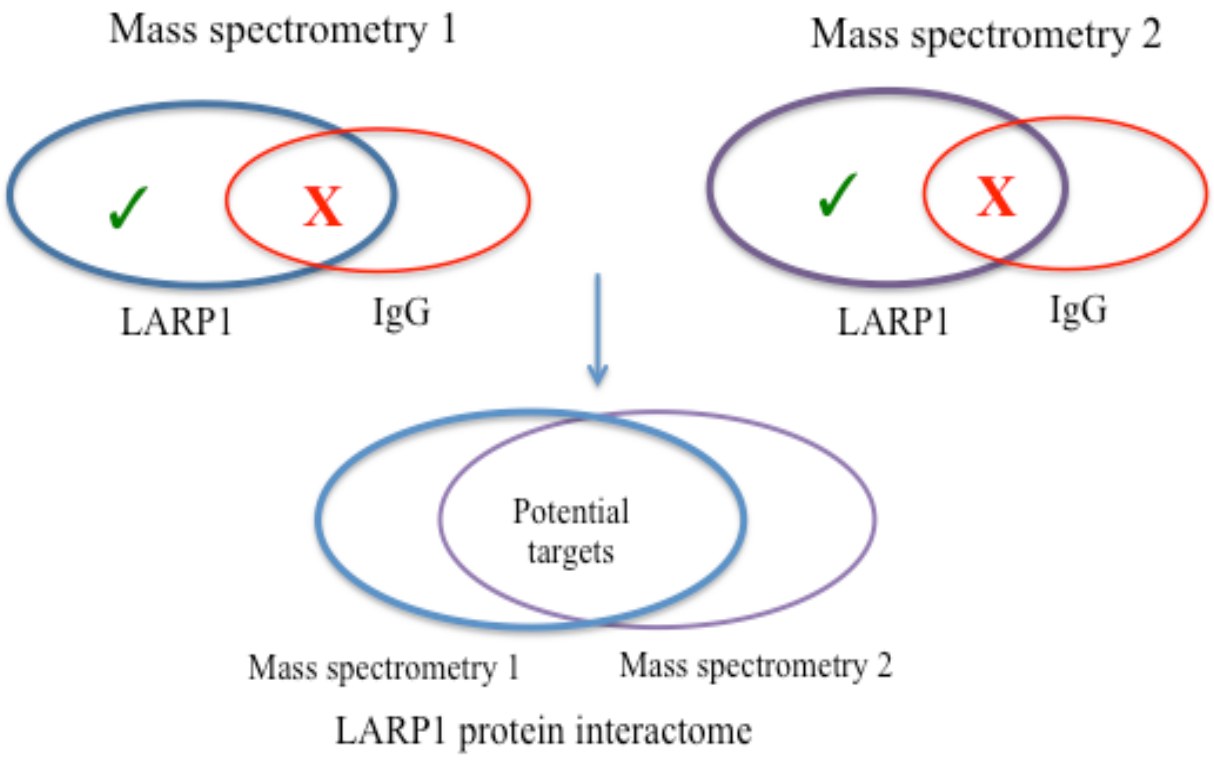


Figure 2-1 Mass spectrometry data interpretation schematic

Schematic showing the process followed in the analysis of the mass spectrometry data to identify the protein interactome as well as the potential protein targets of LARP1.

2.8 INDUCTIVELY COUPLED PLASMA (ICP)-MASS SPECTROMETRY

OVCAR8 cells were transfected with either LARP1 siRNA or control non-targeting siRNA for 48 hours and then treated with cisplatin at a concentration of 25μM for 5h to allow cisplatin to enter the cells and create DNA adducts before triggering the apoptotic cascade. Total DNA was extracted with the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions with a minimum of 1μg of DNA required per sample. Inductively coupled plasma (ICP)-mass spectrometry was used to quantify the amount of cisplatin molecules bound to DNA.

The ICP-MS was an Agilent 7900 equipped with a micromist nebuliser and a cooled spray chamber (Agilent Technologies, USA). The samples were introduced with an ESI SC4 DX autosampler, FAST switching valve and 1 ml sample loop (Elemental Scientific, USA). The ICP-MS and autosampler were controlled with Masshunter 4.3 Workstation Software version C.01.03 (Agilent Technologies, USA).

The assay was calibrated using platinum standards diluted from a 1 mg/L (103 µmol/L) stock (Specure®, Alfa Aesar, Ward Hill, MA) to 2.05 nmol/L, 8.20 nmol/L, 20.50 nmol/L, 82.01 nmol/L, 205.02 nmol/L and 512.56 nmol/L in Milli-Q water (Millipore). Quality Control materials with known platinum concentrations (ClinChek ® Serum Controls, RECIPE, Munich, Germany) were made-up in water as instructed by the manufacturer.

130 µl of each standard, QC material and DNA sample were diluted to a total volume of 4 ml with assay diluent using an automated Microlab 500 Series Diluter/Dispenser (Hamilton, USA). The diluent contained 0.2 % (v/v) ammonia (ROMIL, UK), 0.01% (v/v) Triton X-100 (ROMIL, UK), 0.1% (w/v) EDTA (Sigma Aldrich) and 20 ug/L rhenium (Specure ®, Alfa Aesar, Ward Hill, MA) in Milli-Q water. The ICP-MS carrier and wash solutions were identical to the assay diluent without the addition of rhenium. Each diluted sample was sequentially aspirated into the sample loop and introduced into the ICP-MS nebuliser with the peristaltic pump set to a speed of 0.3 revolutions per second.

Platinum was measured in no-gas mode using mass 195 as the quantifier and mass 185 as the internal standard. Mass 194 was measured as a qualifier. The ICP RF power was set to 1550 W with the tune parameters set to “general purpose”. Each measurement was made at 6 points on the mass peak in triplicate. Calibration curves were constructed in Masshunter by measuring each standard and plotting the CPS (counts per second) of the analyte divided by the CPS of the internal standard against the known standard concentration. All calibrations curves were linear throughout the calibration range. The sample concentrations were

calculated by comparing the analyte CPS divided by the internal standard CPS to the calibration curve.

2.9 NONRADIOACTIVE MEASUREMENTS OF PROTEIN SYNTHESIS WITH SURFACE SENSING OF TRANSLATION (SUNSET)

De novo protein synthesis was measured by Dr Manuela Mura with the use of the surface sensing of translation assay (SuNSET) [346]. OVCAR8 and OVCAR3 cells were cultured in 6–well dishes as described above. Cells when then treated with either cycloheximide 50 ug/ml for 24h, insulin 100 nM for 24h, cisplatin (25μM or 10μM for 24h) or a combination of cisplatin and LARP1 knockdown (24h). Cells were pulsed chased for 15 minutes by incubation with 10 μg/ml of puromycin followed by 15 minutes incubation with normal medium to allow the incorporation of puromycin in nascent peptides. Protein was extracted from the cells and analysed with western blotting using a monoclonal antibody against puromycin (Millipore).

2.10 IMMUNOFLUORESCENCE (IF) STAINING

Cells were cultured on glass coverslips for 24 hours. Genotoxic stress was induced by cisplatin treatment over 24h at an appropriate concentration for each cell line as explained in section 2.1. Cells were washed before being incubated with PHEM fixative (4% PFA, 60mM PIPEs, 25mM HEPES, 10mM EGTA, 4mM MgCl2) for 10 minutes at room temperature. Fixative was removed and cells were washed in PBS and blocked in PBSTB buffer (1% BSA, 0.1% TritonX-100) for 1 hour. Primary antibody solution was applied (Table 2-3) and incubated either for 1h at room temperature or overnight at 4˚C. After washing, Alexa Fluor-conjugated secondary antibodies (Life Technologies) were applied and incubated at room

temperature for 1 hour. Cells were washed and mounted with ProLong Gold mounting medium with DAPI (Life Technologies).

Table 2-3 Antibodies used in immunofluorescence

Antibody	Species	Supplier	Dilution
LARP1	Rabbit	SDIX	1:100
PABP1	Mouse	Abcam	1:100
YB-1	Mouse	Novus biologicals	1:100
PABP4	Mouse	Abnova	1:100
eIF4E	Mouse	Thermo Fisher	1:100

2.11 DUOLINK® USING PLA® TECHNOLOGY

Duolink® protocol was followed as per manufacturer's instructions (Sigma Aldrich, St Louis, MO). Duolink is a method of detecting, quantifying and localising stable or transient protein interactions of protein targets, which are in close proximity (<40nm) and it was used as per manufacturer's instructions. It is based on a proximity ligation assay, which involves secondary antibodies conjugated to oligonucleotide (PLA) probes. The probes form a closed circle that can be ligated if in close proximity. The oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction with the addition of a polymerase. The amplification reaction leads to a high concentration of fluorescence which results in a light spot visualised under confocal microscopy Figure 2-2.

Cells were cultured on glass coverslips for 24 hours. Genotoxic stress was induced by cisplatin treatment over 24h at an appropriate concentration for each cell line. Cells were washed before being fixed and permeabilised with ice-cold methanol. Fixative was removed and cells were washed and blocked in PBSTB buffer (1% BSA, 0.1% TritonX-100) for 1 hour. Primary antibody solution was applied and incubated for 1h at 37˚C. After washing, the Proximity Ligation Assay (PLA) probes were diluted 1:5 in appropriate buffer (as per the

manufacturer's protocol-Sigma-Aldrich) applied and incubated for 60 minutes at 37˚C. Following washing with buffer A (provided by manufacturer), the ligase was diluted in the ligation solution and applied to the samples for 30 minutes at 37˚C. Samples were washed with buffer B and the amplification stock was diluted accordingly and applied to the samples for 100 minutes at 37˚C. Cells were washed in buffer B and mounted with ProLong Gold mounting medium with DAPI (Life Technologies).

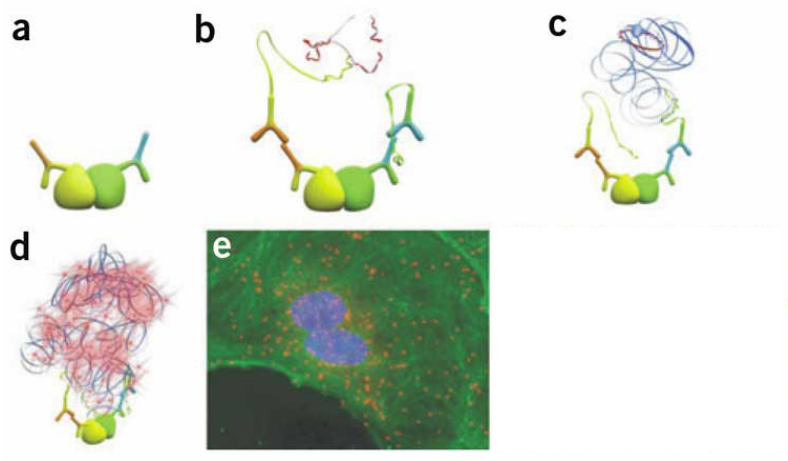


Figure 2-2. Duo-link PLA schematic: visualization of protein-protein interactions

(**a**) Validated primary antibodies for the target proteins raised in two different species are added to the cells); **(b)** Species-specific secondary antibodies are added and each has a different short DNA oligonucleotide strand attached to it (yellow and green, respectively). When the secondary antibodies are in close proximity (<40nm), the DNA strands will hybridise through a subsequent addition of two other circle-forming DNA oligonucleotides (red); **(c)** Enzymatic ligation of the hybridised oligonucleotides leads to their amplification via rolling-circle amplification (RCA) reaction using a polymerase; **(d)** Following the amplification reaction, several hundred replications of the DNA circle have occurred **(e)** The resulting high concentration of fluorescence and each single-molecule amplification product is easily visible as a distinct spot in a fluorescence microscope.

Image adapted and reprinted with permission from Macmillan Publishers Ltd: [Nature Methods] [347], copyright (2010)

2.12 CONFOCAL IMAGING

Immunofluorescence staining as well as the Duo-link interactions were analysed using a Leica 500 confocal microscope and images were processed with Leica LAS AF lite software. All confocal analyses were performed by Dr Manuela Mura.

2.13 TRANSFECTION- TRANSIENT KNOCKDOWN

For transient knockdown, sub-confluent cells were transfected using Dharmafect 1 (GE Dharmacon, Lafayette, CO) according to the manufacturer's instructions, with control non-targeting siRNA 5'->3' (GGUCCGGCUCCCCCAAAUG) and targeting siRNAs for LARP1 (GAAUGGAGAUGAGGAUUGC, AGACUCAAGCCAGACAUCA) and YB1 (GGUCCUCCACGCAAUUACCAGCAAA, GACCCUAUGGGCGUCGACCACAGUA) synthesised by Eurofins (Hamburg, Germany). Transfection mixtures comprised siRNA diluted to a final concentration of 100nM in OptiMEM (GIBCO). In this project, all transient knockdowns were sustained for 48 hours unless stated otherwise.

2.14 THREE-DIMENSIONAL TUMOUR SPHEROID ASSAY

SKOV3 tetracycline inducible (LARP1 shRNA/ GFP shRNA) clones were generated and kindly provided by Dr Katrina Sweeney. Cells were seeded in triplicates in ultra-low adhesion 96-well plates (Corning) at a density of 5 $x10^3$ cells per well with the medium described above. Control clones were named GFP their shRNA sequence targets the green fluorescent protein expressed only in jellyfish (TR30016, Origene). The targeting clone contained an shRNA sequence against LARP1 (TF303581D, Origene). Five days following seeding, the spheroids were formed and were divided in four treatment conditions: 1. No treatment; 2. Tetracycline; 3. Tetracycline plus CDDP started at the same time and 4. Tetracycline followed by CDDP administered 24h later. To achieve LARP1 knockdown

tetracycline was added in the medium at a final concentration of 1μg/mL and CDDP was added at a final concentration of 25μM. They were regularly photographed using a GE ImageQuant LAS 4000 and their area was measured with ImageJ.

2.14.1 TETRACYCLINE INDUCED LARP1 KNOCKDOWN (TET-ON SYSTEM)

The tetracycline-controlled transcriptional activation system (Tet-ON) was used in SKOV3 clones in order to achieve stable LARP1 knockdown [348]. This system uses the antibiotic tetracycline or its derivative doxycycline in order to activate the transcription of a gene of interest (Figure 2-3). A key component of a Tet-ON system is the reverse tetracycline transactivator (rtTA). This protein is created by fusing the reverse Tet repressor (rTet) with the the C-terminal domain of VP16 (virion protein 16) of the Herpes Simplex Virus (HSV) and is capable of binding to the Tetracycline response element (TRE) sequences in the promoter only if bound to tetracycline. Therefore, introduction of tetracycline to the system activates the transcription of the LARP1 shRNA or GFP shRNA accordingly.

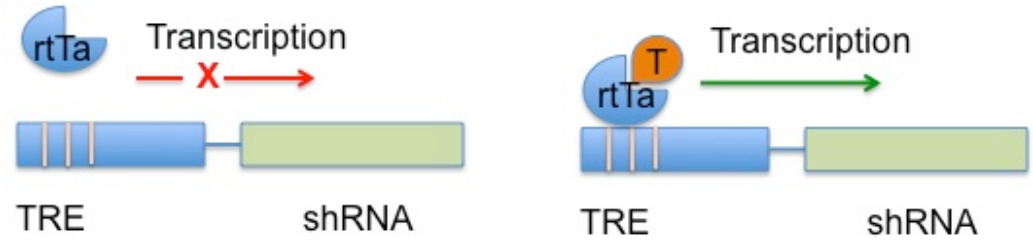


Figure 2-3 TET-ON controlled transcriptional activation system

2.15 XENOGRAFT EXPERIMENT (PILOT STUDY)

The *in vivo* experiment was performed in compliance with the United Kingdom Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986 and within the published guidelines for the welfare and use of animals in cancer research. Female BALB/c nude mice (aged 6–8 weeks; Charles River, Margate, UK) were used. Tetracycline inducible SKOV3 LARP1 shRNA clones and control clones (GFP shRNA) were subcutaneously injected at a concentration of $1x10^6$ in the left and right flank, respectively, of each mouse in a cohort of 4 mice. Tumour dimensions were measured using electronic callipers and tumour volumes calculated by the equation: volume = (π/6) x a x b x c, where a, b, and c represent three orthogonal axes of the tumour. Tumours were classed as measureable when they reached ≥5mm in any axis. Doxycycline diet TD.09295 (Harlan) 1000 ppm was commenced once all mice had formed measurable tumours (~50 mm^3). Intraperitoneal cisplatin was administered 48 hours after doxycycline introduction to a cohort of 2 mice. Cisplatin was injected IP twice a week at a concentration of 1mg/Kg for 2 weeks. The experiment was terminated before any mouse reached pre-set welfare standards were met.

2.16 MTS VIABILITY AND ACTIVATED CASPASE APOPTOSIS ASSAYS

For MTS labelling, 10 x 10^3 cells were cultured at 37°C in 96-well plates with 100µl of media and labelled with 20µl of CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Southampton, UK) and incubated at 37°C. Absorbance at 490nm was recorded on an OPTImax microplate reader (Molecular Devices, Wokingham, UK). MTS assays were performed at the time points specified in Figure legends. Caspase 3/7 activity was assessed using the CaspaseGlo-3/7 Assay (Promega, Southampton, UK). Cells were cultured at 37°C in white opaque 96-well plates (Corning, Ewloe, UK). CaspaseGlo reagent

was added to each well on equal volumes and plates left at room temperature for 1 hour before reading on a LUMIstar Optima plate reader (BMG Labtech, Cambridge, UK). Assays were performed at the time points specified in figure legends.

2.17 STATISTICAL ANALYSIS

Statistical analyses were performed using IBM SPSS statistical package v.22 and GraphPad Prism software (GraphPad Software Inc.). $p \leq 0.05$ was taken to be statistically significant.

2.18 BIOINFORMATIC ANALYSIS

The Search Tool for the Retrieval of Interacting Genes (STRING) database was used to generate interaction networks based on experimentally validated interactions among the identifies LARP1 protein interactors (http://string-db.org). Functional analysis studies were performed using the DAVID bioinformatics database (https://david.ncifcrf.gov) [349] or the Ingenuity Pathway Analysis platform (IPA, http;//www.ingenuity.com).

3 CHAPTER 3- RESULTS

3.1 IDENTIFICATION OF LARP1 INTERACTING PROTEINS WITH A ROLE IN CISPLATIN RESISTANCE

In order to identify LARP1 protein interactors that play a key role in cisplatin resistance, I comparatively explored the LARP1 interactome before and after cisplatin treatment in the cisplatin sensitive (OVCAR3) and resistant (OVCAR8) cell line performing proteomic and functional annotation analyses in each condition. Having observed the differences in the LARP1 complex following cisplatin treatment within each cell line, I then comparatively analysed the differences noted between the two investigated cell lines to identify key proteins that may facilitate the involvement of LARP1 in promoting cisplatin resistance.

3.1.1 PROTEOMIC ANALYSIS OF LARP1 INTERACTOME IN THE CISPLATIN SENSITIVE OVCAR3 CELL LINE

Protein immunoprecipitation was performed using cell lysates of the OVCAR3 cell line as described in the methods section. Cells were treated with cisplatin for 24 hours at a concentration of 10 μM and non-treated cells were used as a control. Immunoprecipitation of the LARP1 complex was confirmed by western blotting Figure 3-1. The LARP1 complex was explored by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) as described in the methods section. The degree of overlap between the two biological repeats of the mass spectrometry in both treatment conditions is rather small Figure 3-2. Pooling together all proteins found in each experimental repeat, a total number of 215 proteins were identified in complex with LARP1 in the untreated cells with only 25 being consistently present in both repeats. Only 73 proteins were identified in total, in the cisplatin treated cells, of which only 15 were found in both repeats.

Such variability may be due to biological and technical reasons. Experimental variations associated with each biological repeat of the experiment are accountable for a degree of variability. Furthermore protein-protein interaction networks are rather dynamic and have an inherent temporal nature [350] therefore a number of ephemeral interactions may have been lost or not captured in one of the experimental repeats. A number of technical parameters need to also be taken into account. There are a number of identified sources of technical variability within the process of quantitative liquid chromatography mass spectrometry (LC-MS) which involve instrumental variance, stability and the process of sample digestion [351].

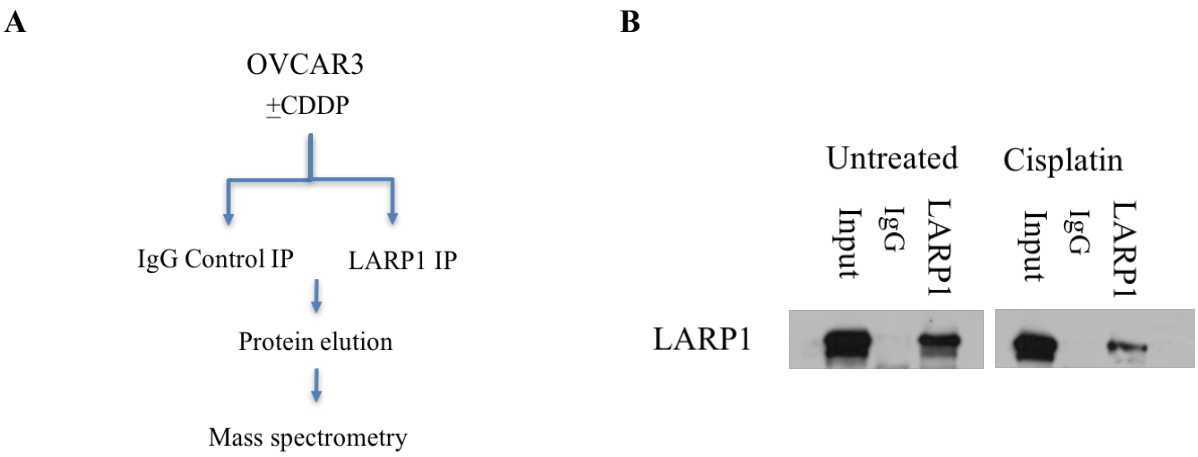


Figure 3-1 LARP1 immunoprecipitation in untreated and cisplatin treated OVCAR3 cells.

A. Schematic showing the protein Immunoprecipitation experiment. Beads were either coated with anti-IgG antibody (negative control) or anti-LARP1 antibody.
B. Western blot of LARP1 protein confirming LARP1 immunoprecipitation. (Input: 10% of the whole cell lysate).

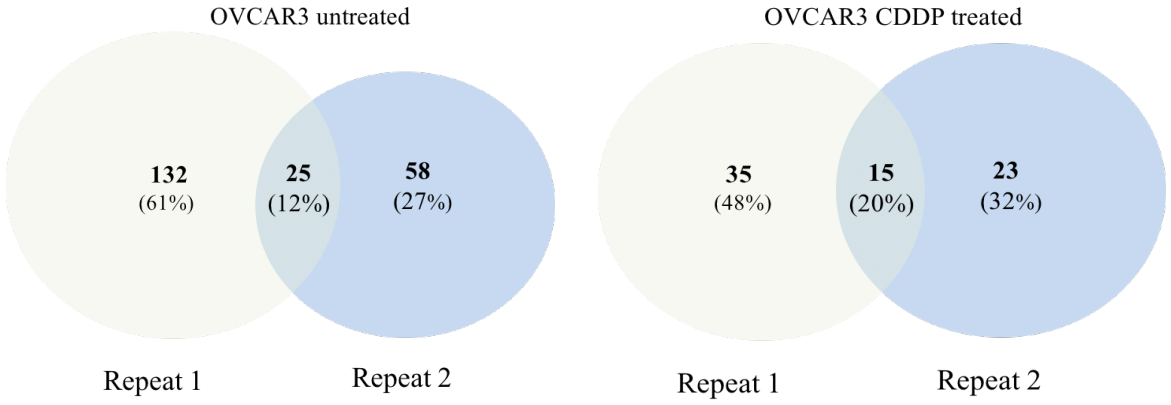


Figure 3-2 Variability of mass spectrometry outcomes for the OVCAR3 cells line.

Venn diagrams showing the degree of overlap between the two biological repeats of the mass spectrometry experiment for the untreated and cisplatin treated OVCAR3 cells.

3.1.1.1 LARP1 protein interactome in untreated OVCAR3 cells

In order to explore and characterise the LARP1 protein complex, I pooled together all the proteins identified in each of the two mass spectrometry repeats and performed proteomic analyses. The LARP1 complex with its 215 interactors in the untreated OVCAR3 cells was visualised with the use of STRING software (http://string-db.org) as seen in Figure 3-3.

LARP1 is found in complex with clusters of proteins involved in protein synthesis (ribosomal proteins 40S and 60S, dead-box helicases, PABP1, YB1) and RNA metabolism (heterogeneous nuclear ribonucleic binding proteins) but also with cytoskeletal proteins associated with cellular movement and architecture (actin, tubulin, myosin). A comprehensive list of all identified interacting proteins is found in Appendix 6.1.1. This list was further curated with more stringent criteria in order to identify only those proteins that were present in both mass spectrometry repeats (Table 3-1).

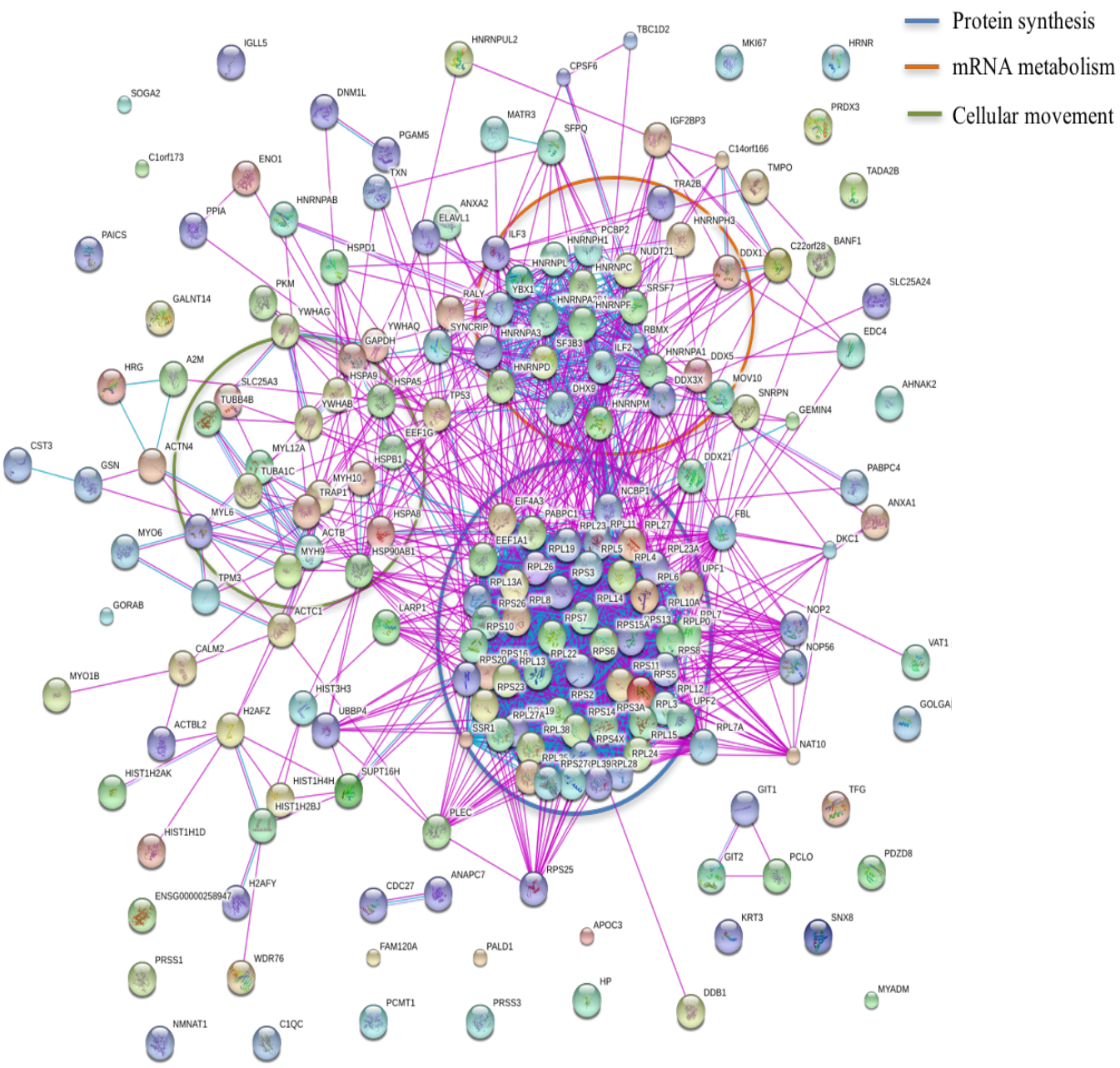


Figure 3-3 LARP1 protein interaction networks in untreated OVCAR3 cells.

STRING network summarising the LARP1 protein complex in the untreated OVCAR3 cell line as identified by UHPLC-MS/MS mass spectrometry. The coloured lines connecting the proteins represent known or predicted interactions based on different levels of evidence. Absence of connecting lines implies no previous evidence for interactions.

Table 3-1 List of proteins in complex with LARP1 in untreated OVCAR3 cells

Gene ID	Accession	Score	Unique peptides identified	Description
AHNK2	Q8IVF2	3359	113	Protein AHNAK2
LAP2A	P42166	2420	74	Lamina-associated polypeptide 2
PABP1	P11940	1428	54	Polyadenylate-binding protein 1
ACTB	P60709	1343	43	Actin, cytoplasmic 1
PABP4	Q13310	1009	46	Polyadenylate-binding protein 4
YBOX1	P67809	603	15	Nuclease-sensitive element-binding protein 1
GRP78	P11021	578	21	8 kDa glucose-regulated protein OS
TUBA1C	F5H5D3	474	14	Tubulin alpha-1C chain
GRP75	P38646	431	13	Stress-70 protein, mitochondrial
H4	P62805	331	10	Histone H4
ROA2	P22626	323	14	Heterogeneous nuclear ribonucleoproteins A2/B1
RS18	P62269	248	9	40S ribosomal protein S18
HNRPU	Q00839	243	9	Isoform Short of Heterogeneous nuclear ribonucleoprotein U
HS90A	P07900	230	7	Isoform 2 of Heat shock protein HSP 90-
NMNA1	Q9HAN9	213	11	Nicotinamide mononucleotide adenyltransferase 1
Q5JR95	Q5JR95	207	7	40S ribosomal protein S8
HORN	Q86YZ3	185	6	Hornerin
RS6	P62753	120	3	40S ribosomal protein S6
RS14	P62263	95	2	40S ribosomal protein S14
KV110	P01602	82	2	Ig kappa chain V-I region HK102
RS23	P62266	75	5	40S ribosomal protein S23
RS25	P62851	67	2	40S ribosomal protein S25
H31T	Q16695	46	3	Histone H3.1t

These proteins were present in both mass spectrometry repeats and absent from the negative IgG isotype.

3.1.1.1.1 Functional annotation for the untreated OVCAR3 cells

To further understand the biological significance of the LARP1 complex, functional annotation analysis was performed using DAVID Bioinformatics Resources v 6.7 [352] and Ingenuity pathway analysis (IPA) software program, (www.ingenuity.com). Eight functional pathways were enriched and listed in Table 3-2.

Table 3-2 Enriched KEGG pathways identified in the LARP1 protein interactome of the untreated OVCAR3 cells

Pathway	Pathway description	Count in gene	False discovery rate
3010	Ribosome	45	**1.68**E-**59**
3040	Spliceosome	11	**3.06**E-**06**
3015	mRNA surveillance pathway	8	0.000126
5203	Viral carcinogenesis	10	0.000528
3103	RNA transport	8	0.00473
5322	Systemic lupus erythematosus	6	0.0114
3018	RNA degradation	5	0.0208

The top five molecular and cellular functions of LARP1 complex are shown in Figure 3-4.

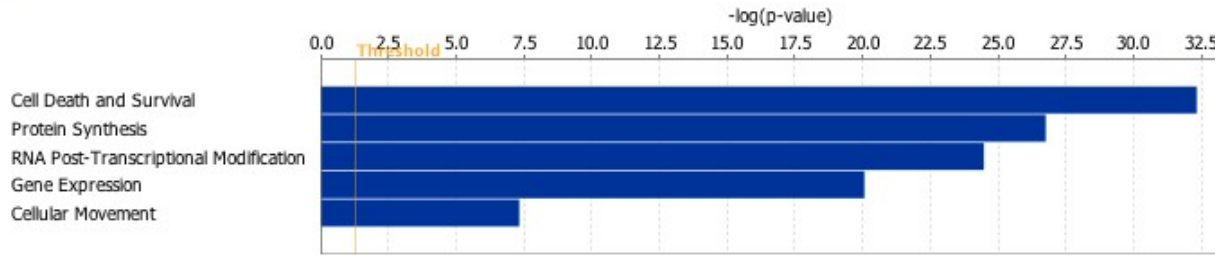


Figure 3-4 Functional enrichment analysis for LARP1 interacting proteins in untreated OVCAR 3 cells.

Top five molecular and cellular functions identified using Ingenuity Pathway Analysis (-log [BH corrected] p-value is shown, yellow dashed line indicates p=0.05).

3.1.1.1.2 Functional annotation for the untreated OVCAR3 cells

Functional analysis shows that in the untreated OVCAR3 cells LARP1 predominantly interacts with proteins involved in protein translation (ribosomal), mRNA stability, transport and metabolism and also cytoskeletal proteins involved in cell movement.

Some of the key proteins involved in each function were clustered by Ingenuity Pathway Analysis and are listed in Table 3-3.

Table 3-3 Representative proteins for the main functional categories identified for LARP1 protein complex in OVCAR3 untreated cells

Protein synthesis	RNA metabolism	Cell death and survival
Ribosomal proteins	HNRNPA1, A2B1, C, D, F, H, L, M	Ribosomal proteins
PABPC1, PABPC4	PABPC1, PABPC4	ANXA1, ANXA2
EIF4A3	DDX5	ENO1
DDX3X, DHX9	CPSF6	PABPC1
ELAV1	RBMX	DDX3X, DDX5, DHX9
YBX1		HSPA9, HSPA90AB1
		ELAVL1
		YBX1

3.1.1.2 LARP1 protein interactome in OVCAR3 cells treated with cisplatin

The same process was also followed for the proteomic analysis of the OVCAR3 cells treated with Cisplatin 10 μM for 24 hours. Pooled mass spectrometry analysis revealed a total of 73 proteins in complex with LARP1 and these are visualised in Figure 3-5.

There is a dramatic decrease in the LARP1 interactome upon genotoxic stress in this cisplatin sensitive cell line, which could be partly explained by the decrease in the number of viable cells upon treatment although equal cell numbers were used in both treatment conditions. Upon cisplatin treatment, LARP1 loses the majority of its binding partners and maintains interaction with mainly ribosomal and cytoskeletal proteins.

A comprehensive list of all identified interacting proteins is included in Appendix 6.1.2. A curated list with the proteins found in both mass spectrometry repeats for the cisplatin treated OVCAR3 cells is found in Table 3-4.

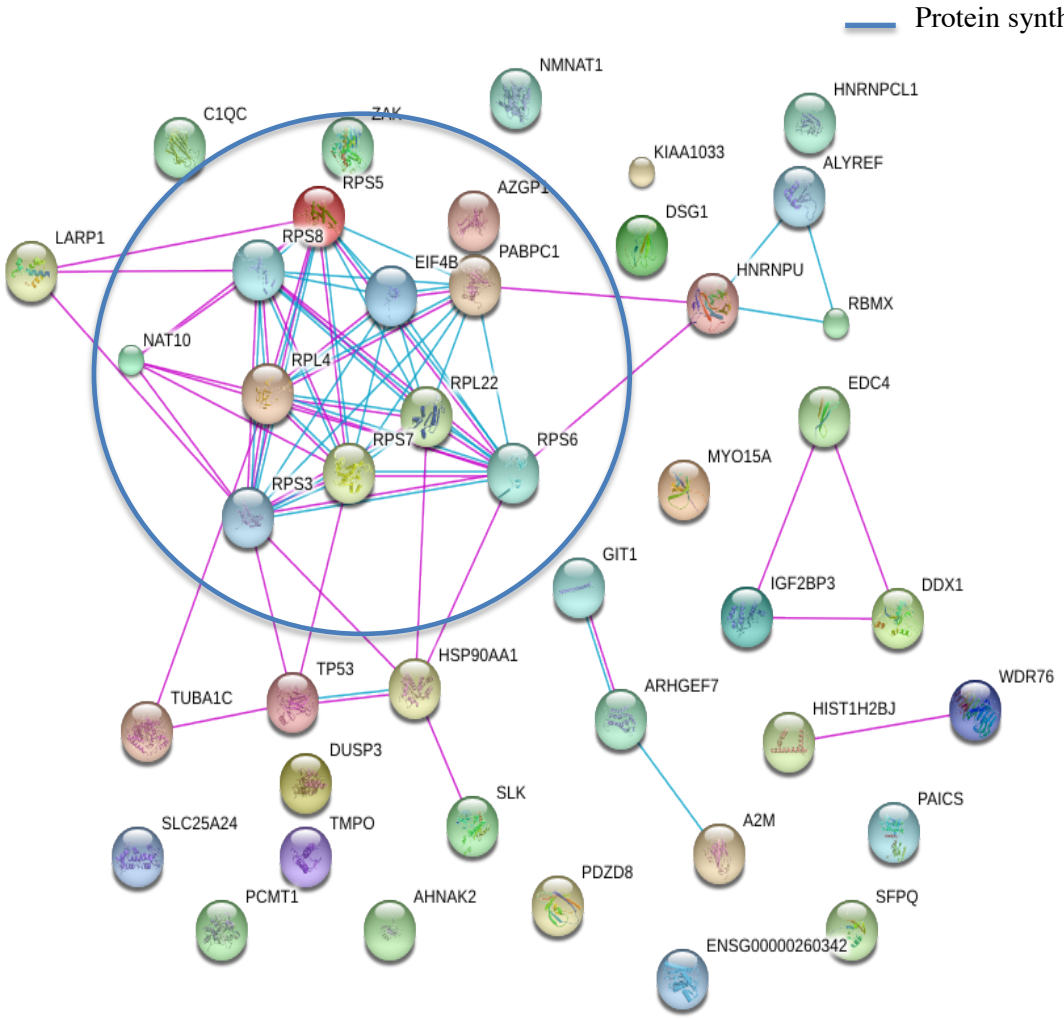


Figure 3-5 LARP1 protein interaction networks in cisplatin treated OVCAR3 cells.

STRING network summarising the LARP1 protein complex in the untreated OVCAR3 cell line as identified by UHPLC-MS/MS mass spectrometry. The coloured lines connecting the proteins represent known or predicted interactions based on different levels of evidence. Absence of connecting lines implies no previous evidence for interactions.

Table 3-4 List of proteins in complex with LARP1 in cisplatin treated OVCAR3 cells

Gene ID	Accession	Score	Unique peptides identified	Description
AHNK2	Q8IVF2	1592	63	Protein AHNAK2
LAP2A	P42166	849	34	Lamina-associated polypeptide 2
NMNA1	Q9HAN9	229	11	Nicotinamide mononucleotide adenyltransferase 1
HSP71	P08107	217	9	Isoform 2 of Heat shock 70 kDa protein 1A/1B
HORN	Q86YZ3	147	6	Hornerin
ACTB	P60709	143	6	Actin, cytoplasmic 1
DSG1	Q02413	124	3	Desmoglein-1
PABP1	P11940	113	3	Polyadenylate-binding protein 1
KV110	P01602	85	2	Ig kappa chain V-I region HK102
FHL2	J3KNW4	69	2	Four and a half LIM domains protein 2
H2B1J	P06899	68	2	Histone H2B type 1-J
PLAK	P14923	64	2	Junction plakoglobin
HS90A	P07900	43	1	Isoform 2 of Heat shock protein HSP 90-alpha

These proteins were present in both mass spectrometry repeats and absent from the negative IgG isotype.

3.1.1.2.1 Functional annotation for the cisplatin treated OVCAR3 cells

Given the small number of proteins remaining in the LARP1 complex upon cisplatin, only one KEGG pathway enriched in the functional analysis and this was for "Ribosome" with a false discovery rate of **1.23**e-^{10}. Four molecular and cellular function ontology enrichments were identified from Ingenuity Pathway Analysis seen in Figure 3-6.

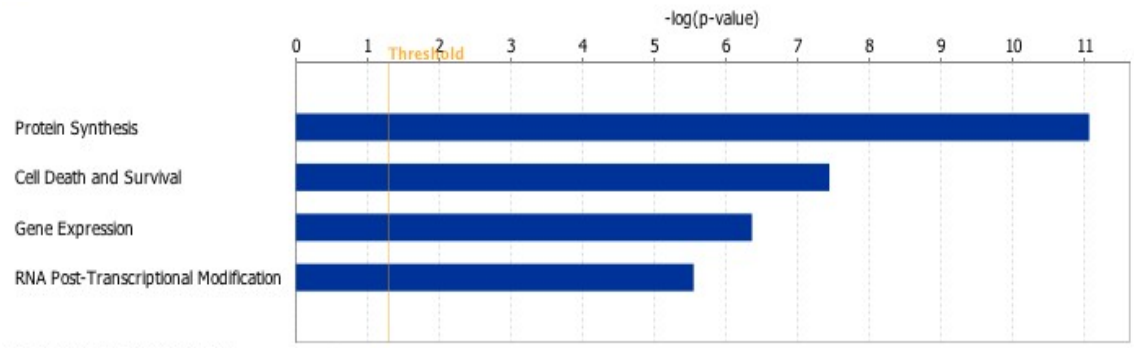


Figure 3-6 Functional enrichment analysis for LARP1 interacting proteins in cisplatin untreated OVCAR 3 cells.

Molecular and cellular functions identified by Ingenuity Pathway Analysis (-log [BH corrected] p-value is shown, yellow dashed line indicates p=0.05).

A selection of proteins representative for each function are listed in Table 3-5.

Table 3-5 Representative proteins for the main functional categories identified for LARP1 protein complex in OVCAR3 cisplatin treated cells.

Protein synthesis	Cell death and survival
Ribosomal proteins	Ribosomal proteins
PABP1	PABP1
EIF4B	A2M

Despite the dramatic change in its complex leading to the loss of the majority of its binding partners, LARP1 is still involved in fundamental cell processes as protein synthesis. This reinforces the previous observation that LARP1 plays a key role in maintaining cell homeostasis.

3.1.1.3 Comparative analysis of LARP1 protein complex in the OVCAR3 cell line before and after cisplatin treatment.

In order to gain a better insight in the changes that occur in the LARP1 complex upon cisplatin treatment, I compared the proteins identified in each treatment condition and performed a functional analysis. This is summarised in Figure 3-7.

In the untreated state, LARP1 is associated with a large complex of proteins involved predominantly in protein synthesis, cell death and survival and RNA metabolism (splicing, mRNA homeostasis, RNA transport, stability and decay). A small fragment of this complex, represented by the overlapping area of the Venn diagram (Figure 3-7) is maintained upon cisplatin in this cell line and mainly consists of ribosomal proteins. Only 36 proteins are uniquely present in the complex upon cisplatin treatment and these are mainly cytoskeletal.

To identify the key proteins associated with LARP1 and its functions in the presence and absence of cisplatin in this cell line, I performed a comparative analysis of the proteins listed in Table 3-1 (protein interactors in untreated OVCAR3 cells) and Table 3-4 (protein interactors in treated OVCAR3 cells). I chose to focus my analysis only on these proteins as I considered them important interactors due to the fact that they were persistently present in both mass spectrometry repeats. The investigated proteins were classified as: 1. those found uniquely in the untreated condition 2. those found uniquely in the treated cells and 3. those found in complex with LARP1 in both the untreated and treated cells. This is summarised in Table 3-6.

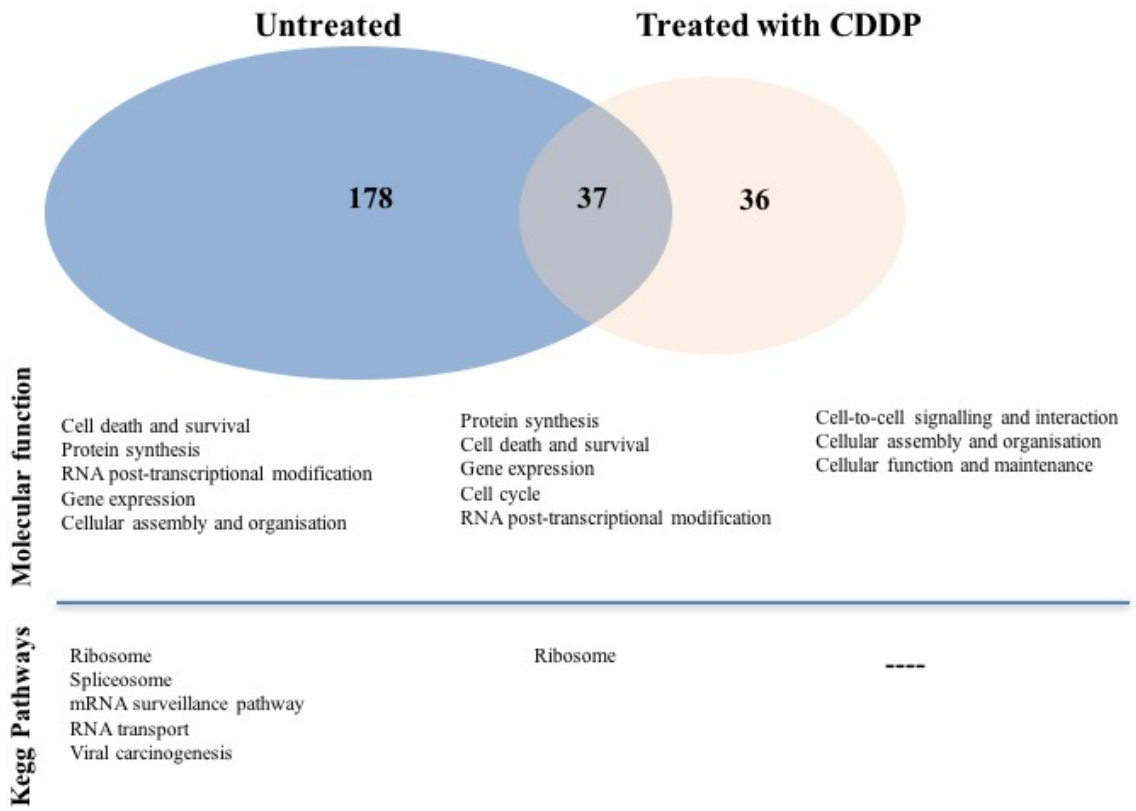


Figure 3-7 Comparative functional analysis of the LARP1 protein interactome in the OVCAR3 cell line before and after treatment with cisplatin.

Venn diagram showing the number of proteins identified uniquely in the untreated cells, the treated cells, or in both conditions. Proteins were clustered according to their function and grouped according to their appearance in the treated, untreated cells or both. A list of the most prominent proteins of each group is provided above.

Table 3-6 Significant LARP1 interactors in the OVCAR3 cells.

Classified according to their presence in complex with LARP1 before or after cisplatin treatment or in both conditions.

Untreated		Untreated &Treated		CDDP treated	
Gene ID	**Accession**	**Gene ID**	**Accession**	**Gene ID**	**Accession**
PABP4	Q13310	**AHNK2**	Q8IVF2	**HSPA71**	P11142
YBOX1	P67809	**LAP2A**	P42166	**DESMOGLEIN 1**	Q02413
GRP78	P11021	**PABP1**	P11940	**FHL2**	J3KNW4
TUBULIN ALPHA 1C	F5H5D3	**ACTIN CYTOPL 1**	P60709	**HISTONE 2B2**	P06899
HISTONE H4	P62805	**HSP90A**	P07900	**PLAKOGLOBIN**	P14923
HNRNPA2B1	P22626	**NMNA1**	Q9HAN9	**KV110**	P01602
RPS18	P62269	**HORNERIN**	Q86YZ3		
HNRNPU	Q00839	**Ig Kappa Chain V-1**	P01602		
RPS6	P62753				
RPS14	P62263				
RPS23	P62266				
RPS25	P62851				
HISTONEH3	Q16695				

Among the "top" LARP1 protein interactors are proteins such as PABP1, PABP4, YB1, AHNK2 and LAP2A. LARP1 maintains its interaction with PABP1, AHNK2 and LAP2A after cisplatin treatment, while losing interaction with PABP4 and YB1. This will be discussed in detail in section 3.1.3.

3.1.2 PROTEOMIC ANALYSIS OF LARP1 INTERACTOME IN THE CISPLATIN RESISTANT OVCAR 8 CELL LINE

Having used the OVCAR3 cell line as a model of cisplatin sensitivity, I concurrently performed the same proteomic analysis in the cisplatin resistant OVCAR8 cell line. Protein immunoprecipitation was performed using cell lysates of the cisplatin resistant cell line OVCAR8 as described in the methods section. Cells were treated with cisplatin for 24 hours at a concentration of 25 μM with non-treated cells being used as a control. Immunoprecipitation of the LARP1 complex was confirmed by western blotting (Figure 3-8). The degree of overlap between the two independent biological repeats of the mass spectrometry is shown in

Figure 3-9 for both treated and untreated OVCAR8 cells.

A grand total of 208 proteins were identified in complex with LARP1 in the untreated OVCAR8 cells of which 49 were uniquely present in the first biological repeat and 137 in the second, with only 22 (10.6%) being present in both. In the cisplatin treated OVCAR8 cells, a total of 194 proteins were identified in the LARP1 complex with 51 uniquely present in the first repeat and 108 in the second. Only 35 (19%) proteins were present in both experimental repeats. Potential factors leading to this variability in the mass spectrometry outcomes were discussed in section 3.1.

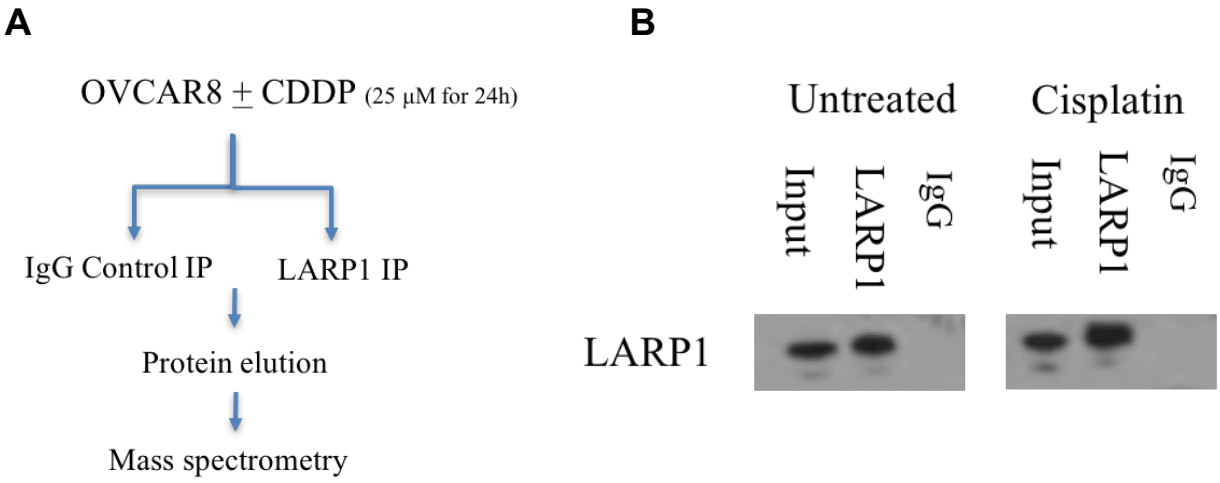


Figure 3-8 LARP1 immunoprecipitation in untreated and cisplatin treated OVCAR8 cells.

A. Schematic showing the protein Immunoprecipitation experiment. Beads were either coated with anti-IgG antibody (negative control) or anti-LARP1 antibody.
B. Western blot of LARP1 protein confirming LARP1 immunoprecipitation. (Input: 10% of the whole cell lysate).

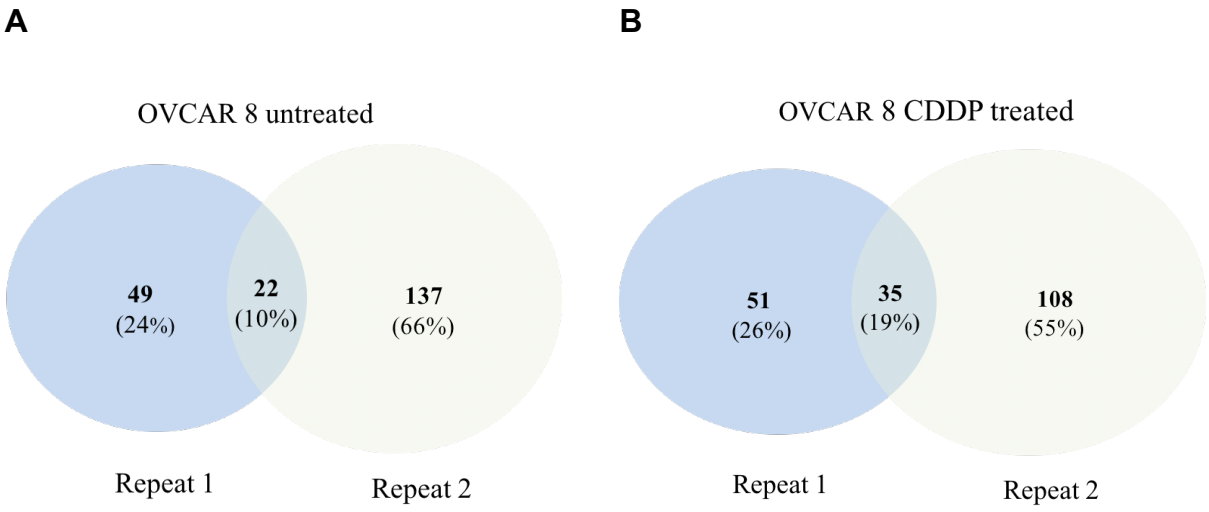


Figure 3-9 Variability of mass spectrometry outcomes for the OVCAR8 cells line.

Venn diagrams showing the degree of overlap between the two biological repeats of the mass spectrometry experiment for the untreated and cisplatin treated OVCAR8 cells.

3.1.2.1 LARP1 protein interactome in untreated OVCAR8 cells

More than 200 proteins were identified as being in complex with LARP1 in the untreated OVCAR8 cells. The identified complex is visualised in Figure 3-10 with the use of STRING software (http://string-db.org).

LARP1 is in complex with clusters of proteins involved in key cellular functions such as protein synthesis (ribosomal proteins 40S and 60S, helicases DDX3, DH9, PABP1, YB1) and mRNA metabolism (heterogeneous nuclear ribonucleic binding proteins). **A** comprehensive list of all identified interacting proteins is found in Appendix 6.1.3. This list was further curated with more stringent criteria in order to identify only those proteins that were present in both mass spectrometry repeats. These are listed in Table 3-7.

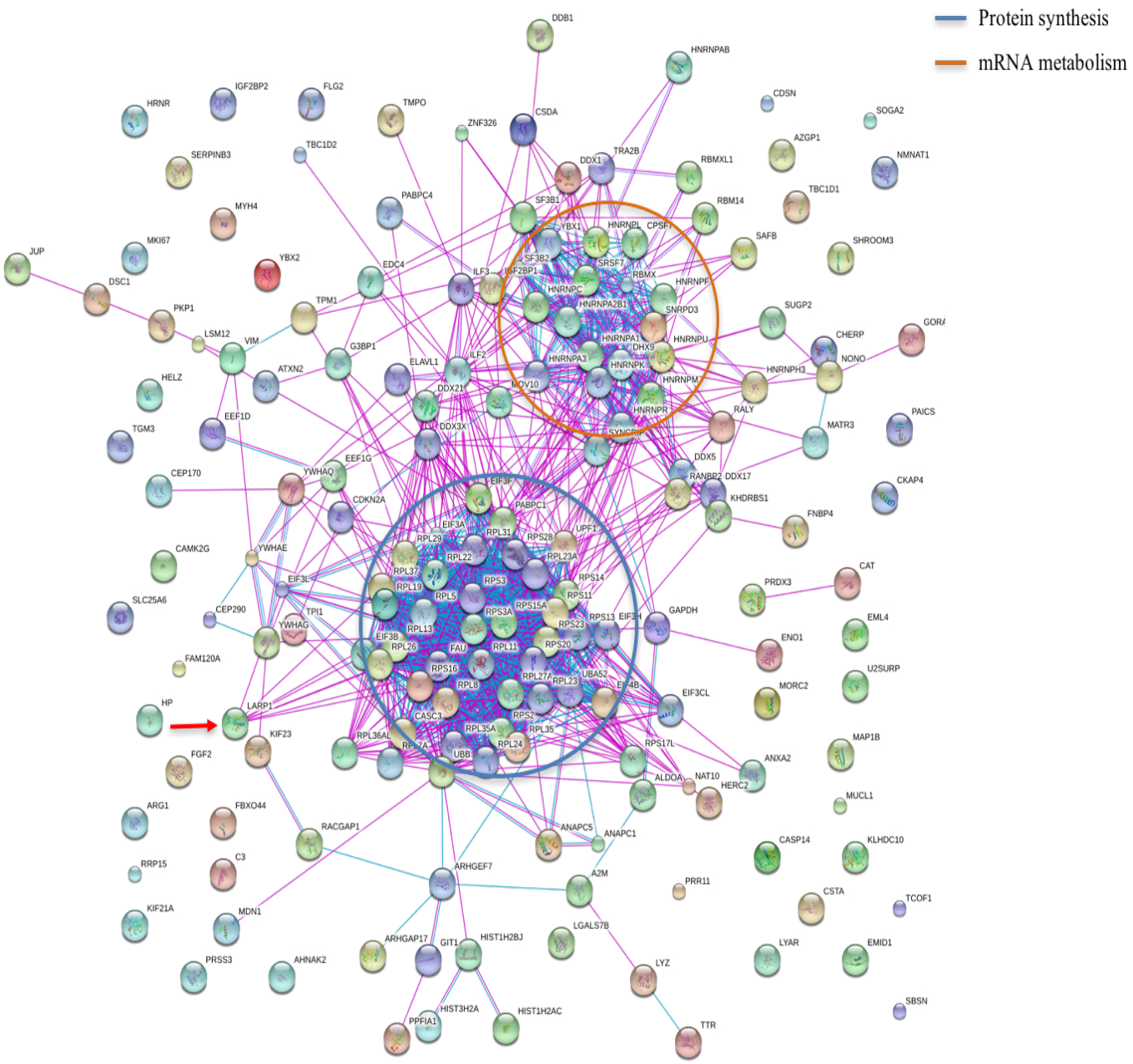


Figure 3-10 LARP1 protein interaction networks in untreated OVCAR8 cells.

STRING network summarising the LARP1 protein complex in the untreated OVCAR8 cell line as identified by UHPLC-MS/MS mass spectrometry. The coloured lines connecting the proteins represent known or predicted interactions based on different levels of evidence. Absence of connecting lines implies no previous evidence for interactions.

Table 3-7 List of proteins in complex with LARP1 in untreated OVCAR8 cells.

Gene name	Accession	Score	Unique peptides identified	Description
LAP2A	P42166	1366	49	Lamina-associated polypeptide 2, isoform alpha
AHNK2	Q8IVF2	1005	44	Protein AHNAK2
PABP1	E7EQV3	860	39	Polyadenylate-binding protein 1
YBOX1	P67809	297	10	Nuclease-sensitive element-binding protein 1
PABP4	Q13310	287	34	Polyadenylate-binding protein 4
TBD2A	Q9BYX2	274	14	TBC1 domain family member 2A
MOV10	Q9HCE1	245	9	Putative helicase MOV-10
A8MXP9	A8MXP9	188	10	Matrin-3
YBOX3	P16989-2	128	6	Isoform 2 of Y-box-binding protein 3
CE170	Q5SW79	118	7	Centrosomal protein of 170 kDa
MTCL1	Q9Y4B5	117	6	Microtubule cross-linking factor 1
HNRPU	Q00839	111	3	Heterogeneous nuclear ribonucleoprotein U
CATA	P04040	87	3	Catalase
DSC1	Q08554	80	3	Desmocollin-1
NAT10	Q9H0A0	71	4	N-acetyltransferase 10
CASPE	P31944	63	2	Caspase-14
F120A	Q9NZB2-6	52	3	Isoform F of Constitutive coactivator of PPAR-gamma-like protein 1
EDC4	Q6P2E9	45	4	Enhancer of mRNA-decapping protein 4
F8VV32	F8VV32	38	2	Lysozyme C
CDSN	G8JLG2	38	1	Corneodesmosin
RPL35	F2Z388	27	1	60S ribosomal protein L35
RBP2	P49792	26	1	E3 SUMO-protein ligase RanBP2

These proteins were present in both mass spectrometry repeats and absent from the negative IgG isotype.

3.1.2.1.1 Functional annotation for the untreated OVCAR8 cells

To further understand the biological significance of the LARP1 complexes, functional annotation analysis was performed as described earlier for the OVCAR3 cell line (section 3.1.1.1.1). Pathway analysis for the identified proteins revealed 8 enriched KEGG pathways as shown in Table 3-8.

Table 3-8 Enriched KEGG pathways identified from LARP1 protein interactome in the untreated OVCAR8 cells.

Pathway ID	Pathway description	Count in gene set	False discovery rate (FDR)
3010	Ribosome	31	6.05E-34
3040	Spliceosome	14	5.78E-10
3013	RNA transport	11	8.50E-06
5203	Viral carcinogenesis	9	0.00218
4114	Oocyte meiosis	6	0.0181
4110	Cell cycle	6	0.0321
3015	mRNA surveillance pathway	5	0.0381
5322	Systemic lupus erythematosous	5	0.0497

To further implement the analysis, I explored the molecular and cellular functions of the identified proteins with IPA software with the top 5 functions shown in Figure 3-11.

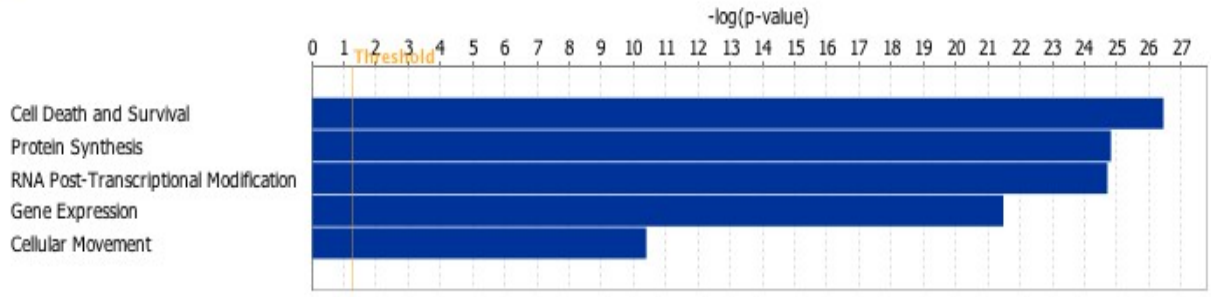


Figure 3-11 Functional enrichment analysis for LARP1 interacting proteins in untreated OVCAR8 cells.

Top 5 Molecular and cellular functions identified by Ingenuity Pathway Analysis (-log [BH corrected] p-value is shown, yellow dashed line indicates p=0.05).

Functional analysis confirmed what was observed in Figure 3-10 and consistently demonstrated that LARP1 complex is predominantly involved in protein synthesis, mRNA metabolism (folding/unfolding, modification, stabilisation, splicing and maturation) and also cell death/ survival. The key proteins involved in each function are listed in Table 3-9.

Table 3-9 Representative proteins for the main functional categories identified for LARP1 protein complex in OVCAR8 untreated cells

Protein Synthesis	RNA metabolism	Cell death and survival
Ribosomal proteins	HNRNP A1, A2B1, C, F, H3, L, U	Ribosomal proteins
PABPC1	AHNAK	ATXN2
EIF3F, EIF3H, EIF3B	DDX17, DDX5, DDX21	A2M
DDX3X, DHX9	NONO	ENOA1
YBX1, YBX2	RBMX	EIF3F, EIF3B, EIF3L
CASC3	YBX1, YBX2	PABPC1
	PABPC1	CAT
		MAP1B
		DDX5, DDX17, DHX9
		YBX1, YBX2, YBX3
		DDB1

Identified by Ingenuity Pathway Analysis

3.1.2.2 LARP1 interactome in OVCAR8 cells treated with cisplatin

The same analysis process was also followed for the OVCAR8 cells treated with Cisplatin (at a concentration of 25 μM for 24 hours). Pooled mass spectrometry analysis revealed a total of 194 proteins in complex with LARP1 and these are visualised in Figure 3-12.

Again, LARP1 is found to interact with proteins mainly involved in protein synthesis and RNA metabolism as also seen in the untreated cells. A comprehensive list of all interacting proteins as is found in Appendix 6.1.4. The curated list of the proteins that were identified in both biological repeats of the mass spectrometry are listed in Table 3-10.

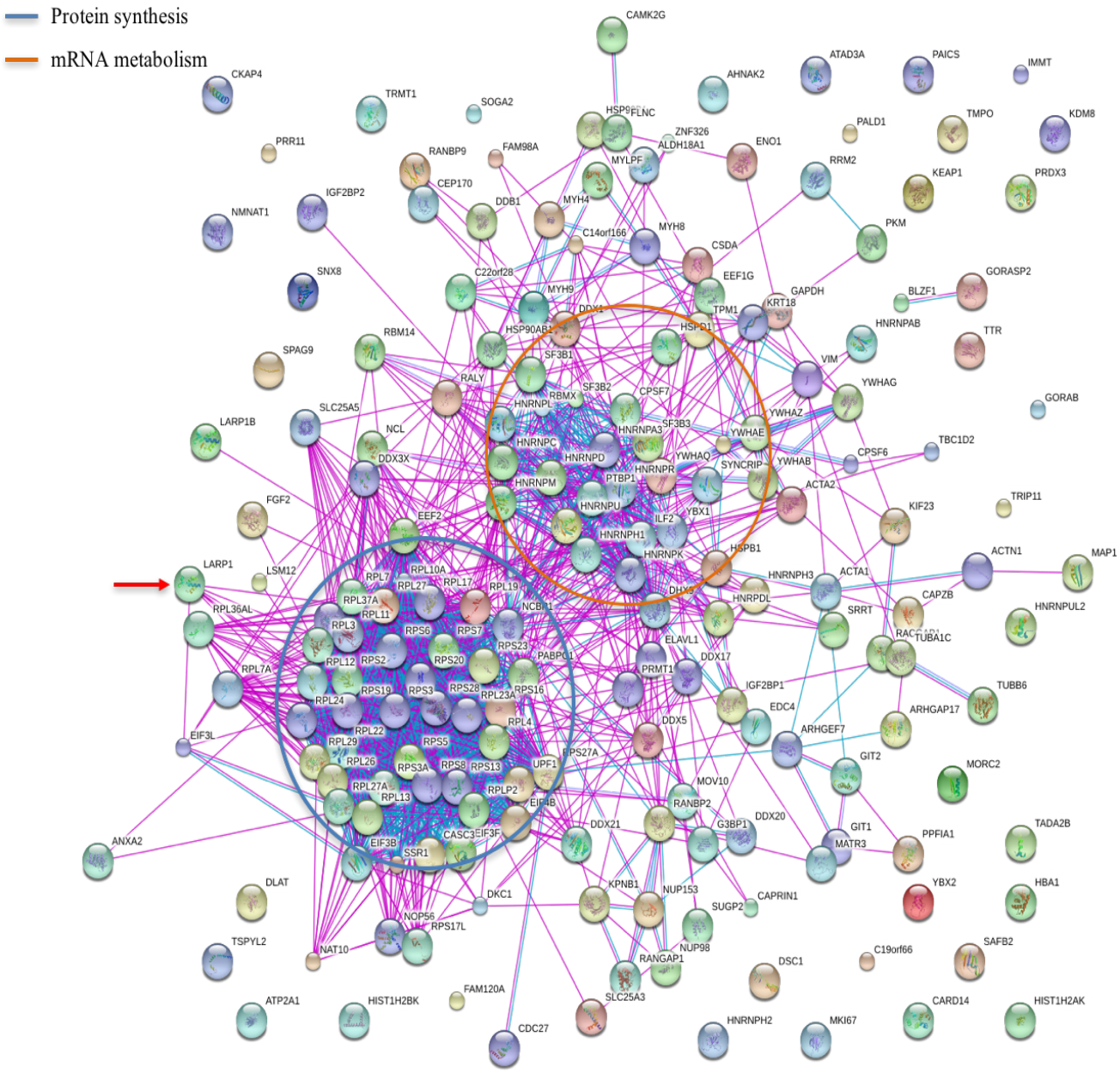


Figure 3-12 LARP1 protein interaction networks in OVCAR8 cells treated with cisplatin 25 µM over 24h.

STRING network summarising the LARP1 protein complex in the untreated OVCAR8 cell line as identified by UHPLC-MS/MS mass spectrometry. The coloured lines connecting the proteins represent known or predicted interactions based on different levels of evidence. Absence of connecting lines implies no previous evidence for interactions.

Table 3-10 List of proteins in complex with LARP1 in OVCAR8 cells treated with cisplatin

Gene ID	Accession	Score	Unique peptides identified	Description
LAP2A	P42166	1687	75	Lamina-associated polypeptide 2, isoform alpha
PABPC1	P11940	1298	61	Polyadenylate-binding protein 1
AHNK2	Q8IVF2	1250	52	of Protein AHNAK2
MTCL1	Q9Y4B5	1105	48	Microtubule cross-linking factor 1
CE170	Q5SW79	984	42	Centrosomal protein of 170 kDa
PABP4	Q13310	850	72	Polyadenylate-binding protein 4
YBOX1	P67809	492	13	Nuclease-sensitive element-binding protein 1
SNX8	Q9Y5X2	436	14	Sorting nexin-8
TUBA1C	F5H5D3	334	12	Tubulin alpha-1C chain
MOV10	Q5JR04	315	14	Mov10, Moloney leukemia virus 10
HNRPL	P14866	268	7	Heterogeneous nuclear ribonucleoprotein L
YBOX2	Q9Y2T7	257	7	Y-box-binding protein 2
PALD	Q9ULE6	219	6	Paladin
HNRPM	P52272	201	12	Heterogeneous nuclear ribonucleoprotein M
RENT1	Q92900	197	11	Regulator of nonsense transcripts 1
FAM98A	Q8NCA5	192	5	Protein FAM98A
EDC4	Q6P2E9	163	9	Enhancer of mRNA-decapping proteinC4
HNRPU	Q00839	159	6	Heterogeneous nuclear ribonucleoprotein U
HNRPH	G8JLB6	145	4	Heterogeneous nuclear ribonucleoprotein H
RTCB	Q9Y3I0	138	7	tRNA-splicing ligase RtcB homolog
DDX5	J3KTA4	126	6	Probable ATP-dependent RNA helicase DDX5
KIF23	B4E1K0	125	3	Kinesin-like protein
ZNF326	Q5BKZ1	105	5	DBIRD complex subunit ZNF326
DDX3X	O00571	104	4	Isoform 2 of ATP-dependent RNA helicase
CH60	P10809	87	3	60 kDa heat shock protein, mitochondrial
DDX21	Q9NR30	68	1	Isoform 2 of Nucleolar RNA helicase 2
RGAP1	Q9H0H5	67	1	Rac GTPase-activating protein 1
ENOA	P06733	62	1	Isoform MBP-1 of Alpha-enolase
IF2B1	Q9NZI8	59	3	Insulin-like growth factor 2 mRNA-binding protein 1
HNRPQ	O60506	55	2	Heterogeneous nuclear ribonucleoprotein Q
MAP1B	P46821	53	3	Microtubule-associated protein 1B
EF1G	P26641	48	1	Isoform 2 of Elongation factor 1-gamma
GORAB	Q5T7V8	34	1	Isoform 2 of RAB6-interacting golgin

These proteins were present in both mass spectrometry repeats.

3.1.2.2.1 Functional annotation for the treated OVCAR 8 cells

Functional annotation analysis was performed as described before with 11 KEGG pathways being enriched as listed in Table 3-11.

Table 3-11 Enriched KEGG pathways identified in the LARP1 protein interactome of the cisplatin treated OVCAR8 cells

Pathway ID	Pathway description	Count in gene set	False discovery rate (FDR)
3010	Ribosome	34	5.07E-38
3013	RNA transport	13	2.23E-07
3040	Spliceosome	11	2.04E-06
5203	Viral Carcinogenesis	11	7.21E-05
5130	Pathogenic Eserichia coli	6	0.000472
4114	Oocyte meiosis	7	0.00261
3015	mRNA surveillance pathway	6	0.006
4110	Cell cycle	6	0.0329
4151	Pi3K-Akt signaling pathway	10	0.0338
4530	Tight junction	6	0.0338
5169	Epstein Barr virus infection	7	0.05

The top five molecular and cellular functions of LARP1 complex are shown in Figure 3-13.

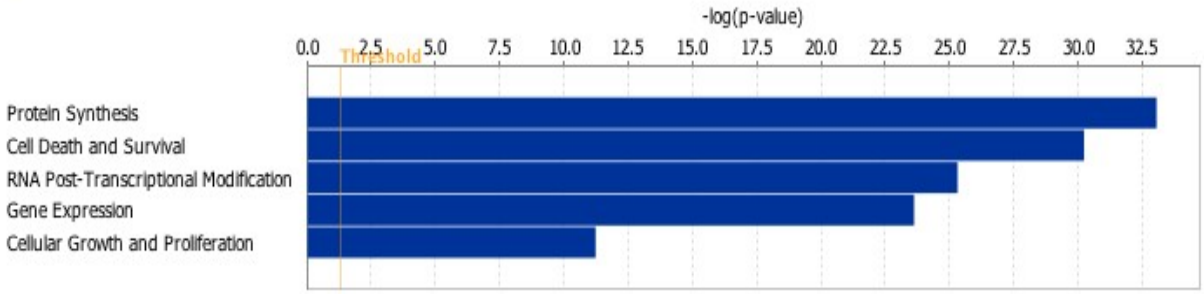


Figure 3-13 Functional enrichment analysis for LARP1 interacting proteins in cisplatin treated OVCAR8 cells.

Top 5 molecular and cellular functions identified by Ingenuity Pathway Analysis (-log [BH corrected] p-value is shown, yellow dashed line indicates p=0.05).

LARP1 remains involved in protein synthesis and RNA metabolism upon cisplatin treatment, maintaining its interaction with proteins belonging to the same functional categories as in the untreated cells. However new functional pathways appeared, including the Pi3K-Akt signalling pathway, which plays a pivotal role in cell proliferation and survival [353].
Furthermore, LARP1 is now also complexed with proteins associated with cellular growth and proliferation which indicates that it is involved in key protein networks that maintain cell survival upon cisplatin treatment in this resistant cell line. Some of the key proteins involved in each function were clustered by Ingenuity Pathway Analysis and are listed in Table 3-12.

Although LARP1 remains in complex with clusters of proteins that are involved in protein synthesis and RNA metabolism, the individual components of these complexes are not identical in the two conditions (treated vs untreated). One example is that heat shock proteins are only complexed with LARP1 in the treated cells. Furthermore, the heterogeneous nuclear ribonucleic binding proteins K (HNRNPK) is in complex with LARP1 in the cisplatin treated cells but not in the control sample. This shows a number of dynamic changes occurs in order for the cells to preserve these fundamental cellular functions upon genotoxic stress [354].

Table 3-12 Representative proteins for the main functional categories identified for LARP1 protein complex in OVCAR8 untreated cells.

Protein synthesis	RNA metabolism	Cell death and survival
Ribosomal proteins	HNRNPA1, A2B1, C, F, H3, K, L, M, U	Ribosomal proteins
PABPC1, PABPC4	ANHAK	Heat shock proteins
EIF3B, 3IF2B, EIF3L, IEF3F	PABPC1, PABPC4	ANXA2
DDX3X, DHX9	DDX17, DDX20, DDX5, DDX21	ENO1
ELAV1	YBX2	EIF3B, EI3F, EIF3L
YBX1, YBX2	ELAV1	PABPC1
HSPA5, HSPB1		PRMT1
		YBX1, YBX2
		DDX3, DDX5, DDX17, DDX20, DHX9
		CMK2G

3.1.2.3 Comparative analysis of LARP1 protein complex in the OVCAR8 cell line before and after cisplatin treatment.

As previously described for the OVCAR3 cell line, I also compared the proteins identified in each treatment condition and performed a functional analysis for the OVCAR8 cell line. This is summarised in Figure 3-14.

A total of 108 unique proteins were found in complex with LARP1 in the untreated cells and 121 in the treated. In both conditions LARP1 interacts with a "stable complex" of 100 proteins. Upon cisplatin treatment LARP1 preserves a core complex which remains unchanged reflecting that LARP1 remains involved in fundamental cellular processes such as protein synthesis, RNA metabolism and cell survival regardless the presence of genotoxic stress. Following cisplatin treatment, LARP1 also dynamically forms new protein interactions and new functional pathways arise, such as the Pi3K-Akt signalling pathway, showing the involvement of LARP1 in cell survival during genotoxic stress. Whether LARP1 holds an active or passive role in the re-arrangement of its protein interactions and consequently the cellular functions it gets involved to upon cisplatin is uncertain.

In the untreated OVCAR8 cells LARP1 is mainly in complex with ribosomal proteins, whereas in the cisplatin-treated cells it seems to predominantly interact with proteins involved in splicing, mRNA homeostasis, RNA transport, stability and decay. This could be an indicator that in state a of genotoxic stress LARP1 may affect splicing or even mediate the transport of transcripts that are necessary for the cells to adapt to the new conditions. It could also reflect the fact that upon genotoxic stress the transcription shifts into a "new" state with new transcripts being available and RNA binding proteins consequently re-arrange their targets and complexes.

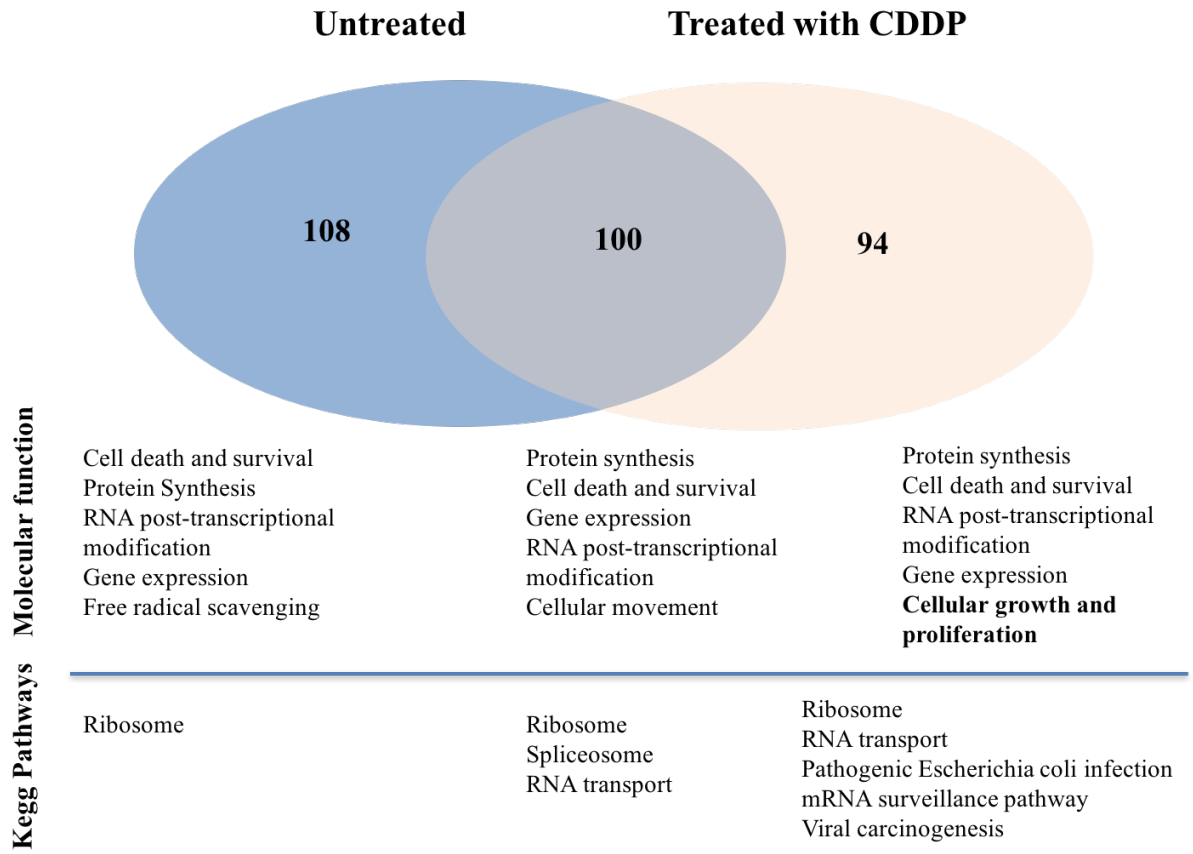


Figure 3-14 Comparative functional analysis of the LARP1 protein interactome in the OVCAR8 cell line before and after treatment with cisplatin.

Venn diagram showing the number of proteins identified uniquely in the untreated cells, the treated cells, or in both conditions. Proteins were clustered according to their function and grouped according to their appearance in the treated, untreated cells or both. A list of the most prominent proteins of each group is provided above.

In order to identify the key proteins associated with LARP1 and its functions in the presence and absence of cisplatin, I performed a comparative analysis of the proteins listed in Table 3-7 (protein interactors in untreated OVCAR8 cells) and Table 3-10 (protein interactors in treated OVCAR8 cells). As also mentioned earlier I focused my analysis only on these proteins as they were persistently present in both mass spectrometry biological repeats. The investigated proteins were classified as: 1. those found uniquely in the untreated condition 2. those found uniquely in the treated cells and 3. those found in complex with LARP1 in both the untreated and treated cells. These are presented in Table 3-13 and ranked according to their Mascot score.

Table 3-13 Significant LARP1 interactors in the OVCAR8 cells.

Classified according to their presence in complex with LARP1 before or after cisplatin treatment or in both conditions.

Untreated		Untreated &Treated		CDDP treated	
Name	**Accession**	**Name**	**Accession**	**Name**	**Accession**
TBD2A	Q9BYX2	**AHNK2**	Q8IVF2	**SORTING NEXIN 8**	Q9Y5X2
MATRIN 3	A8MXP9	**LAP2A**	P42166	**FILAMIN C**	Q14315
YBOX3	P16989	**PABP1**	P11940	**TUBULIN ALPHA**	F5H5D3
FILAGGRIN	Q5D862	**YBOX1**	P67809	**HNRNPL**	P14866
GAPDH	P04406	**PABP4**	Q13310	**YBOX2**	Q9Y2T7
CATALASE	P04040	**HNRNP**	Q00839	**AHNK**	Q09666
ANNEXIN2	H0YMD0	**MTCL1**	Q9Y4B5	**PALADIN**	Q9ULE6
NAT 10	Q9H0A0	**CE170**	Q5SW79	**HNRNPM**	P52272
CASPASE 14	P31944	**MOV 10**	Q5JR04	**RENT1**	Q92900
FAM120A	Q9NZB2	**EDC4**	Q6P2E9	**FAM98A**	Q8NCA5
LYSOZYME	F8VV32			**HNRNPH**	Q6P2E9
CORNEODESMOSIN	G8JLG2			**RTCB**	Q8NCA6
RPL35	F2Z388			**DDX5**	J3KTA4
RANBP2	P49792			**KIF23**	B4E1K0
				DBIRD COMPLEX	Q5BKZ1
				DDX3	B4E1K2
				HSPD1	P10809
				RACGAP1	Q9H0H5
				DDX21	Q9NR30
				ALPHA ENOLASE	P06733
				IGF2BP	Q9NZI8
				HNRNPQ	O60506
				ELONGATION FACTOR 1	P26641
				GORAB	Q5T7V8

3.1.3 KEY LARP1 PROTEIN INTERACTORS WITH A ROLE IN CISPLATIN RESISTANCE

The proteomic analysis of LARP1 interactome in the cisplatin resistant (OVCAR8) and the sensitive (OVCAR3) cell line shows that, in the untreated condition of both cell lines, LARP1 is in complex with a large number of proteins and might be involved in fundamental cellular processes including protein translation, mRNA metabolism and cell survival. A key difference between the two examined cell lines is noted upon cisplatin treatment. In the resistant cell line, LARP1 maintains a "stable" large protein complex and also obtains a number of new binding partners. Functional analysis demonstrated that, upon genotoxic stress, LARP1 not only preserves its cellular functions but is also involved in new pathways associated with cell proliferation. On the contrary, in the cisplatin sensitive cell line, LARP1 maintains only a small fraction of its pre-existing protein complex while losing the majority of its protein interactors, suggesting a change in its functional role which is mainly confined to protein synthesis.

In order to identify LARP1 protein interactors that play a role in cisplatin resistance, I performed a comparative analysis of the proteins found as significant interactors in each of the examined cell lines (Table 3-6 and Table 3-13). The proteins listed in these tables were evaluated on the basis on their mass spectrometry scores and their biological relevance with cisplatin resistance.

In both investigated cell lines, the proteins AHNAK2, LAP2A, PABP1, YB1 and PABP4 appeared among the "top" LARP1 protein interactors.

Both AHNAK and LAP2A have structural functions and are located in the nucleus. LAP2A is predominantly involved in maintaining the structural organisation of nuclear lamina [355] and AHNAK2 belongs to a family of scaffold PDZ proteins implicated in membrane repair [356], though its exact cellular function has not yet been clearly defined. Despite their consistently high scores in the mass spectrometry experiments, these proteins were not

further evaluated as their functions are predominantly structural with no links to cisplatin resistance.

Poly (A) binding protein 1 (PABPC1) has been well described for its key role in mRNA translation, stability and decay [357, 358]. It is predominantly cytoplasmic but also found to shuttle in the nucleus implying its involvement in nucleocytoplasmic mRNA transport [359]. PABPC1 has been associated with carcinogenesis. It is upregulated in gastric cancer correlating with poor survival outcomes [360] and also overexpressed in high-grade hepatocellular carcinoma where it enhances anchorage independent growth [361].

YB-1 is a multifunctional protein involved in transcription, splicing stability and translation of several mRNAs [362-364]. It is involved in DNA repair mediating the cellular response to genotoxic stress via activation of the human multidrug resistance gene (MDR1) [365] and has also been found to promote strand separation of duplex DNA containing either mismatches or cisplatin modifications [366]. YB-1 is predominantly cytoplasmic but translocates to the nucleus upon ultraviolet irradiation [367] and its nuclear expression has been associated with adverse survival outcomes and drug resistance in breast [368], ovarian [369], squamous cell lung cancer[370] and sarcomas [371]. Furthermore YB-1 has been implicated in cisplatin resistance in breast cancer via its protein interactions[372].

PABPC4 (or iPABP) is another member of the PABP family, first identified in activated T cells [373] and also as an antigen on the surface of thrombin-activated platelets [374]. It is located primarily in the cytoplasm but can also shuttle in the nucleus [375] [376] and has been closely related to PABPC1 but has no established function in translation [373]. PABPC4 is highly expressed in colorectal cancer and associated with better prognosis [377]. Furthermore, it has been found to be enriched in docetaxel resistant prostate cancer cell derived exosomes [378] and to regulate cell growth in endometrial cancer cell lines [379].

In the untreated state of both examined cell lines, LARP1 interacts with PABP1, YB1 and PABP4. However, upon cisplatin treatment, this complex is only preserved in the resistant cell line, whereas only interaction with PABP1 is maintained in the sensitive cell line. This observation generated the hypothesis that this complex may play a key role in maintaining cell homeostasis and survival upon genotoxic stress and its further investigation could unveil a possible mechanism for cisplatin resistance.

PABP1, PABP4 and particularly YB1 were identified as key LARP1 protein targets in the OVCAR8 cell line and were brought forward for further validation

3.1.4 SUMMARY

In summary, in this first results section I explored the protein interactome of LARP1 protein in the cisplatin sensitive (OVCAR3) and resistant (OVCAR8) cell line before and after cisplatin treatment. In both cell lines LARP1 was found in complex with a reproducible cluster of proteins involved in fundamental cell processes including protein translation, mRNA metabolism and transport (ribosomal, hnRNPs, heat shock proteins). Upon cisplatin-induced genotoxic stress, LARP1 changed some of its binding partners maintaining its cellular functions in the OVCAR8 cells, whereas it lost the majority of its interactors in the OVCAR3 cell line. I identified PABP1, PABP4 and YB1 as target proteins which interact with LARP1 and have been involved in carcinogenesis and cisplatin resistance. These interactions are maintained in the OVCAR8 but disrupted in the OVCAR 3 cell line upon cisplatin treatment and this may underpin a potential mechanism for cisplatin resistance.

3.2 TARGET VALIDATION

3.2.1 VALIDATION OF LARP1-PABP1, LARP1- PABP4 AND LARP1-YB1 INTERACTION WITH IMMUNOPRECIPITATION FOLLOWED BY WESTERN BLOTTING

As a first step towards the validation of the identified key binding partners of LARP1 (section 3.1), I immunoprecipitated the LARP1 complex in the OVCAR3 and OVCAR8 cell lines (as described in section 3.1) and analysed it by western blotting for the proteins PABP1, PABP4 and YB1 Figure 3-15.

The mass spectrometry findings were confirmed in OVCAR3 cell line with all three proteins being present in the untreated cells and only PABP1 being in complex with LARP1 upon cisplatin Figure 3-15 (A).

In regards to the OVCAR8 cell line, western blotting confirmed the mass spectrometry finding of PABP1 and YB1 as components of the LARP1 complex in both untreated and treated OVCAR8 cells. However, PABP4 was only identified in the untreated cells Figure 3-15 (B). Since PABP4 was identified in both mass spectrometry biological repeats in treated and untreated cells with a relatively high Mascot score, this inconsistency between the mass spectrometry and the western blot validation could imply that there may have been a change involving its epitope following cisplatin treatment. A change in the binding partners of PABP4 upon cisplatin may have obstructed the binding of the used anti-PABP4 antibody to its epitope. However, the same result was observed despite using two different anti-PABP4 antibodies (mono- and polyclonal). Another speculation is that cisplatin could have potentially caused conformational changes to PABP4 protein which could have affected the epitope(s) recognised by the antibodies. In fact, cisplatin has been known to bind to proteins causing changes in their molecular structure [380, 381] .

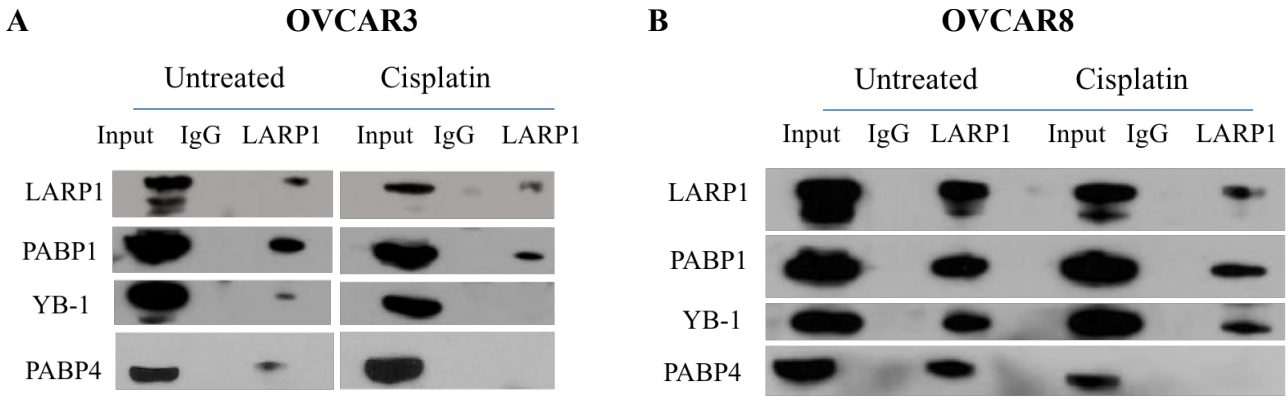


Figure 3-15 Western blot of LARP1 immunoprecipitation stained for its PABP1, PABP4 and YB1

A. OVCAR cell line; B. OVCAR8 cell line

Validation of the interaction between LARP1 and the protein targets selected from liquid chromatography tandem mass spectrometry (LC-MS/MS) in the OVCAR3 and OVCAR8 cell lines in the presence and absence of cisplatin treatment. Proteins PABP1, YB1 and PABP4 were validated. The eluent from the protein A sepharose beads was analysed by western blotting with antibodies against the indicated proteins. Input: whole cell lysate; IgG: eluent from IgG covered beads; LARP1: eluent from LARP1 covered beads

3.2.2 VALIDATION BY REVERSE IMMUNOPRECIPITATIONS AND WESTERN BLOTTING

A further step for the validation of key LARP1 interactors was to perform reverse immunoprecipitations using as bait antibodies against the protein of interest (PABP1, PABP4, YB1) and anti-IgG antibody as a negative control. The precipitated protein complex for each investigated antibody was analysed for the presence of LARP1 by western blotting.

3.2.2.1 OVCAR3 cell line

In the cisplatin sensitive OVCAR3 cell line LARP1 was precipitated in the PABP1, PABP4 and YB1 individual complexes in the untreated cells Figure 3-16. Upon treatment, LARP1 was not present in any of its targets' complexes. This was expected for PABP4 and YB1 based on the mass spectrometry data and the initial validation Figure 3-15 (A). However, LARP1 was not precipitated in the PABP1 complex (anti-PABP1 immunoprecipitation Figure 3-16 (A) despite the fact that PABP1 was precipitated in the LARP1 complex (anti-LARP1 immunoprecipitation) as seen in Figure 3-15 (A). I wondered whether the anti-PABP1 antibody was sharing the epitope with the site of interaction between LARP1 and PABP1 (if the interaction between PABP1 and LARP1 proteins was direct) and repeated the experiment with different anti-PABP1 antibodies both monoclonal and polyclonal obtaining the same outcome. However, later on I show that the interaction between LARP1 and PABP1 is RNA-dependent. Therefore, I speculate that probably a cisplatin–induced conformational change in either PABP1 or LARP1 or both, could have affected the outcome of this reverse immunoprecipitation.

3.2.2.2 OVCAR8 cell line

LARP1 was precipitated in the PABP1 and YB1 protein complexes respectively in both the untreated and cisplatin treated OVCAR8 cells Figure 3-17 (A, C). However, it was present in the PABP4 complex only in the untreated cells Figure 3-17 (B). This observation is consistent with Figure 3-15 (B) but inconsistent with the previous mass spectrometry findings were PABP4 was found in complex with LARP1 in both treatment conditions and potential reasons for this have already been discussed in section 3.2.1

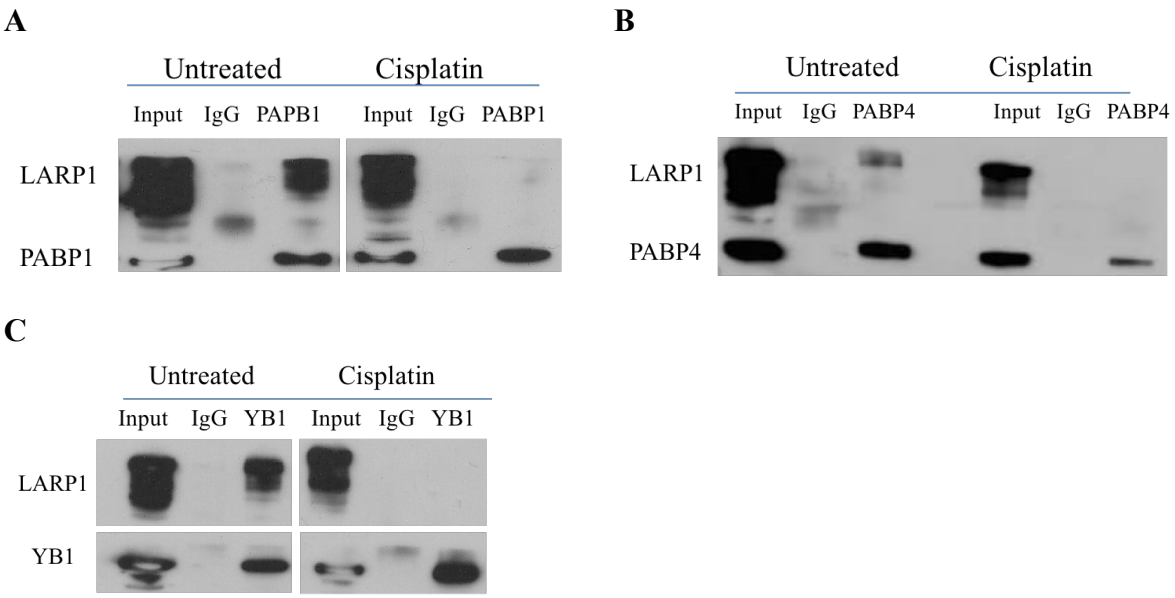


Figure 3-16 Western blots of the reverse immunoprecipitations of target proteins analysed for LARP1 in OVCAR3 cells.

Reverse immunoprecipitations (IPs) using anti-PABP1 **(A)**, anti-PABP4 **(B)** and anti-YB1 antibody **(C)** as baits to precipitate LARP1.

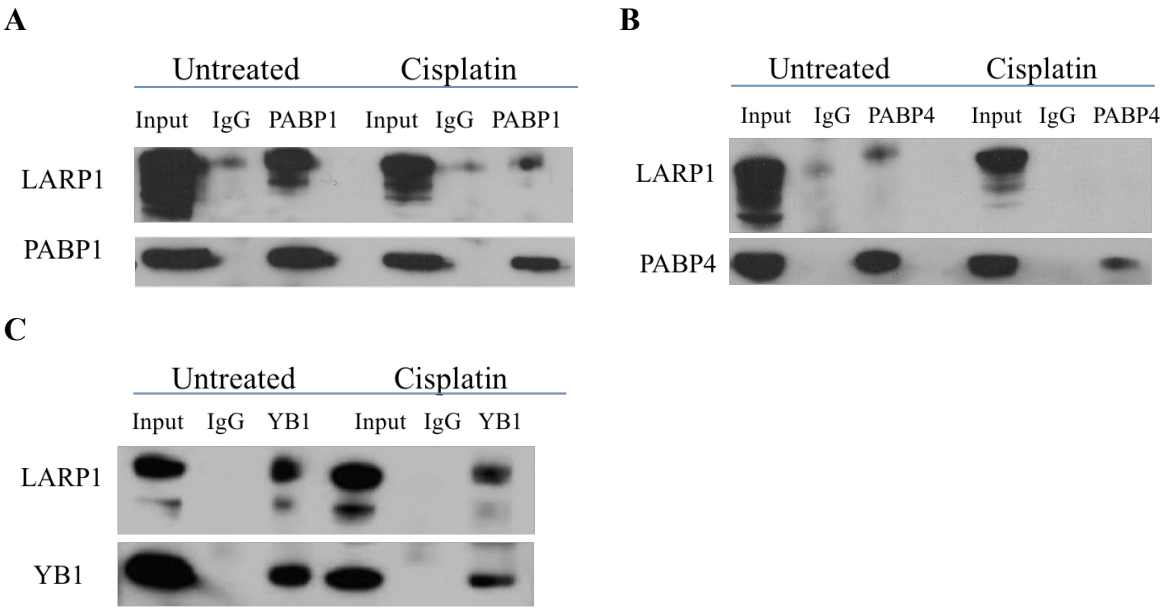


Figure 3-17 Western blots of the reverse immunoprecipitations of target proteins analysed for LARP1 in OVCAR8 cells.

Reverse immunoprecipitations (IPs) using anti-PABP1 (A), anti-PABP4 (B) and anti-YB1 antibody (C) as baits to precipitate LARP1

3.2.2.3 Summary

In order to validate the key LARP1 protein targets identified from the mass spectrometry, I performed reciprocal immunoprecipitations for PABP1, PABP4 and YB-1 and analysed each precipitated complex for the presence of LARP1 by western blotting. I confirmed that PABP1, PABP4 and YB1 are all in complex with LARP1 in the untreated OVCAR8 cells but only the interactions with PABP1 and YB1 were confirmed upon cisplatin treatment. This discrepancy in the PABP4-LARP1 interaction is speculated to have occurred due to cisplatin-induced conformational changes in either of the proteins. In regards to the OVCAR3 cell line, LARP1 is confirmed to be in complex with PABP1, PABP4 and YB-1 in the untreated cells with only the interaction with PABP1 remaining post-treatment.

3.2.3 LOCALISATION STUDIES - IMMUNOFLUORESCENCE

Immunofluorescence was used in order to investigate the subcellular localisation of LARP1 as well as its colocalisation with its target proteins (PABP1, PABP4, YB1) upon cisplatin treatment in sensitive and resistant cell lines.

3.2.3.1 Subcellular localisation of LARP1 before and after cisplatin (CDDP) treatment

The subcellular localisation of LARP1 has been previously investigated in our lab in the cisplatin resistant cell line PEO4. Despite being also present in the nucleus and particularly at the nuclear rim, LARP1 is predominantly cytoplasmic in resting cells but becomes markedly nuclear upon treatment with cisplatin Figure 3-18. Intracellular translocation has been reported for other RNA binding proteins such as HuR in response to stress [382]. Protein LARP3 is known to undergo C-terminal cleavage resulting to its nuclear localisation [383] but a post-translational modification for LARP1 has not yet been thoroughly investigated.

I questioned whether nuclear accumulation of LARP1 following genotoxic stress could be a characteristic of cisplatin resistant cell lines and potentially a way to mediate cell survival upon genotoxic stress. To answer this, I explored the subcellular localisation of LARP1 in two sensitive and resistant cell lines. As models of cisplatin resistance, I used the OVCAR8 and CP70 cell lines and for sensitivity the OVCAR3 and A2780 cell lines.

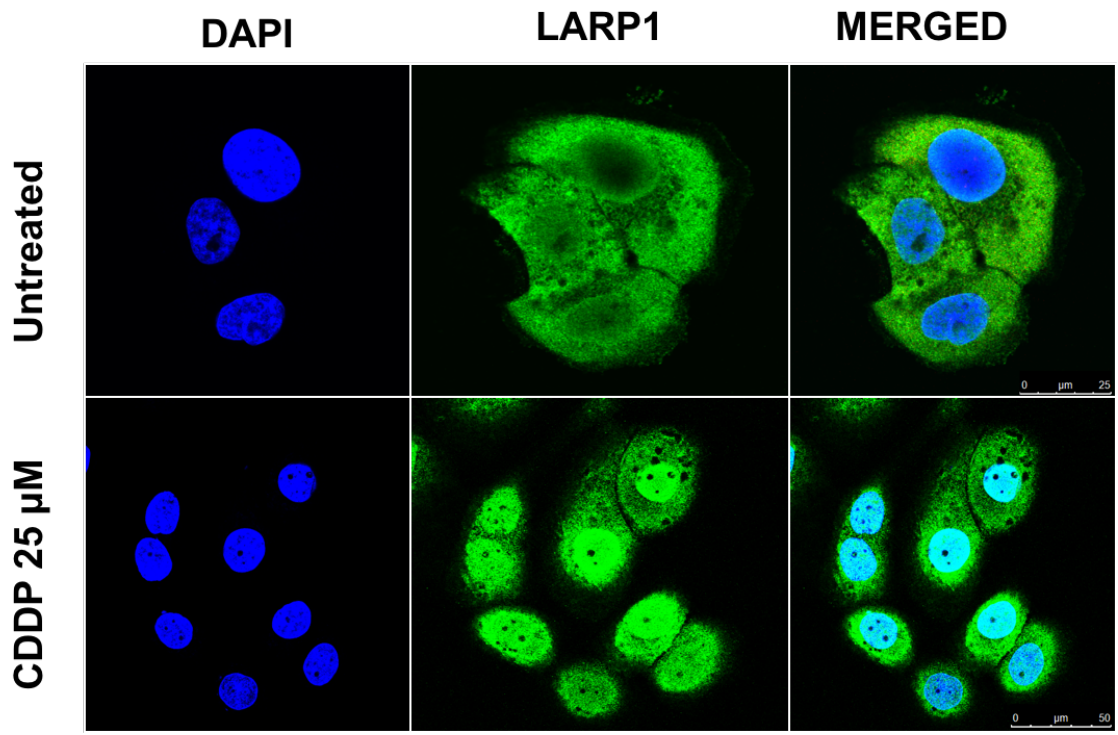


Figure 3-18 LARP1 localisation in PE04 cells before and after treatment with cisplatin.

LARP1 is cytoplasmic in untreated cells and becomes nuclear after cisplatin treatment in the cisplatin resistant PEO4 cell line. (Data from Dr Manuela Mura)

3.2.3.1.1 LARP1 localisation in cisplatin resistant cell lines

As seen in Figure 3-18, Figure 3-19 and Figure 3-20, in the investigated cisplatin resistant cell lines (PE04, OVCAR8 and CP70 respectively) LARP1 is enriched in the nucleus after cisplatin treatment compared to its predominantly cytoplasmic appearance in the untreated state.

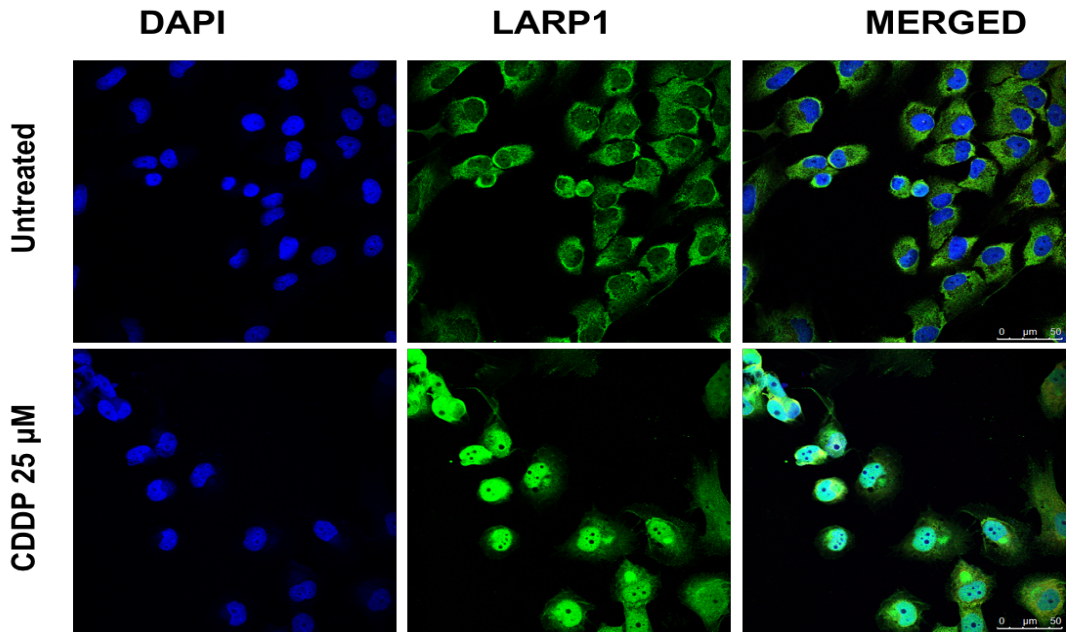


Figure 3-19 LARP localisation in OVCAR8 cells before and after treatment with cisplatin.

Immunofluorescence confocal microscopy of LARP1 (green) with DAPI counterstaining (blue) in untreated OVCAR8 cells, or following 24 hours exposure to 25µM CDDP.

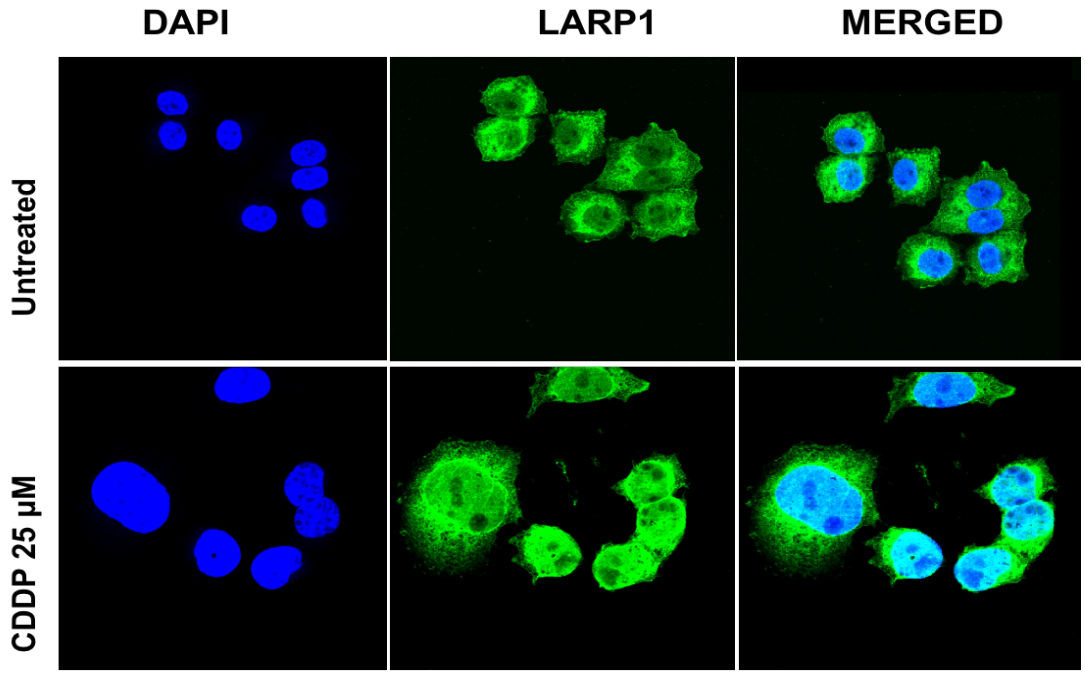


Figure 3-20 LARP localisation in CP70 cells before and after treatment with cisplatin.

Immunofluorescence confocal microscopy of LARP1 (green) with DAPI counterstaining (blue) in untreated OVCAR8 cells, or following 24 hours exposure to 25µM CDDP.

3.2.3.1.2 LARP1 localisation in cisplatin sensitive cell lines

I repeated the experiment in the cisplatin sensitive cell lines OVCAR3 and A2780.

The same pattern was also observed in the examined cisplatin sensitive cell lines Figure 3-21 and Figure 3-22 . This indicates the nuclear localisation of LARP1, induced by cisplatin, is not a unique characteristic of cisplatin resistant cell lines but perhaps associated with the fundamental cellular functions of LARP1.

Having found that LARP1 becomes nuclear following treatment with cisplatin in all the investigated cell lines, I wished to establish whether this nuclear localisation was specific to cisplatin induced genotoxic damage. Therefore, I repeated the experiment using other chemotherapeutic agents such as paclitaxel, gemcitabine and bleomycin. While bleomycin has a similar mechanism of action to cisplatin, causing DNA damage, paclitaxel and gemcitabine do not damage the DNA [7]. I identified that only bleomycin caused nuclear localisation of LARP1 whereas upon treatment with the other chemotherapeutic agents it remained cytoplasmic Figure 3-23, suggesting that its nuclear translocation is triggered by genotoxic stress.

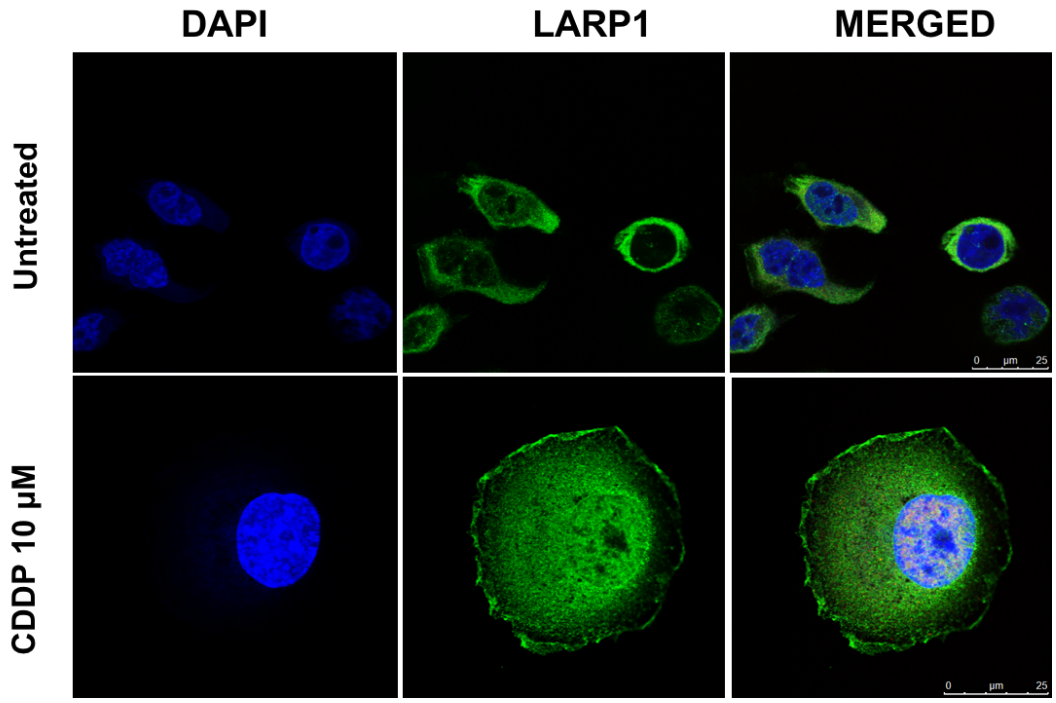


Figure 3-21 LARP1 localisation in OVCAR3 cells before and after treatment with cisplatin.

Immunofluorescence confocal microscopy of LARP1 (green) with DAPI counterstaining (blue) in untreated OVCAR8 cells, or following 24 hours exposure to 10 µM CDDP.

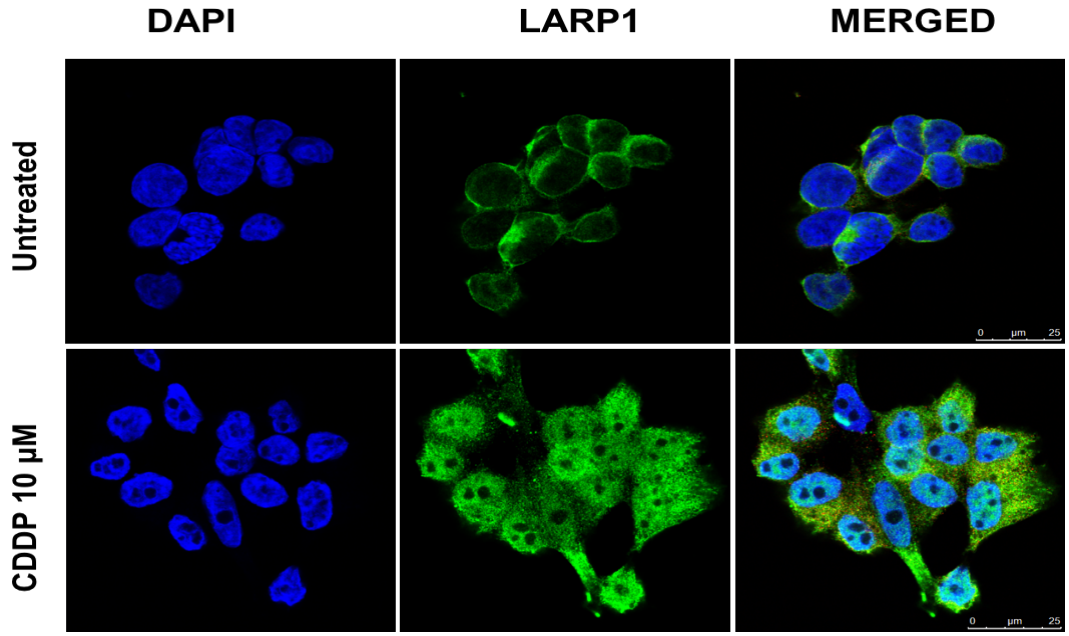


Figure 3-22 LARP1 localisation in A2780 cells before and after treatment with cisplatin.

Immunofluorescence confocal microscopy of LARP1 (green) with DAPI counterstaining (blue) in untreated OVCAR8 cells, or following 24 hours exposure to 10 µM CDDP.

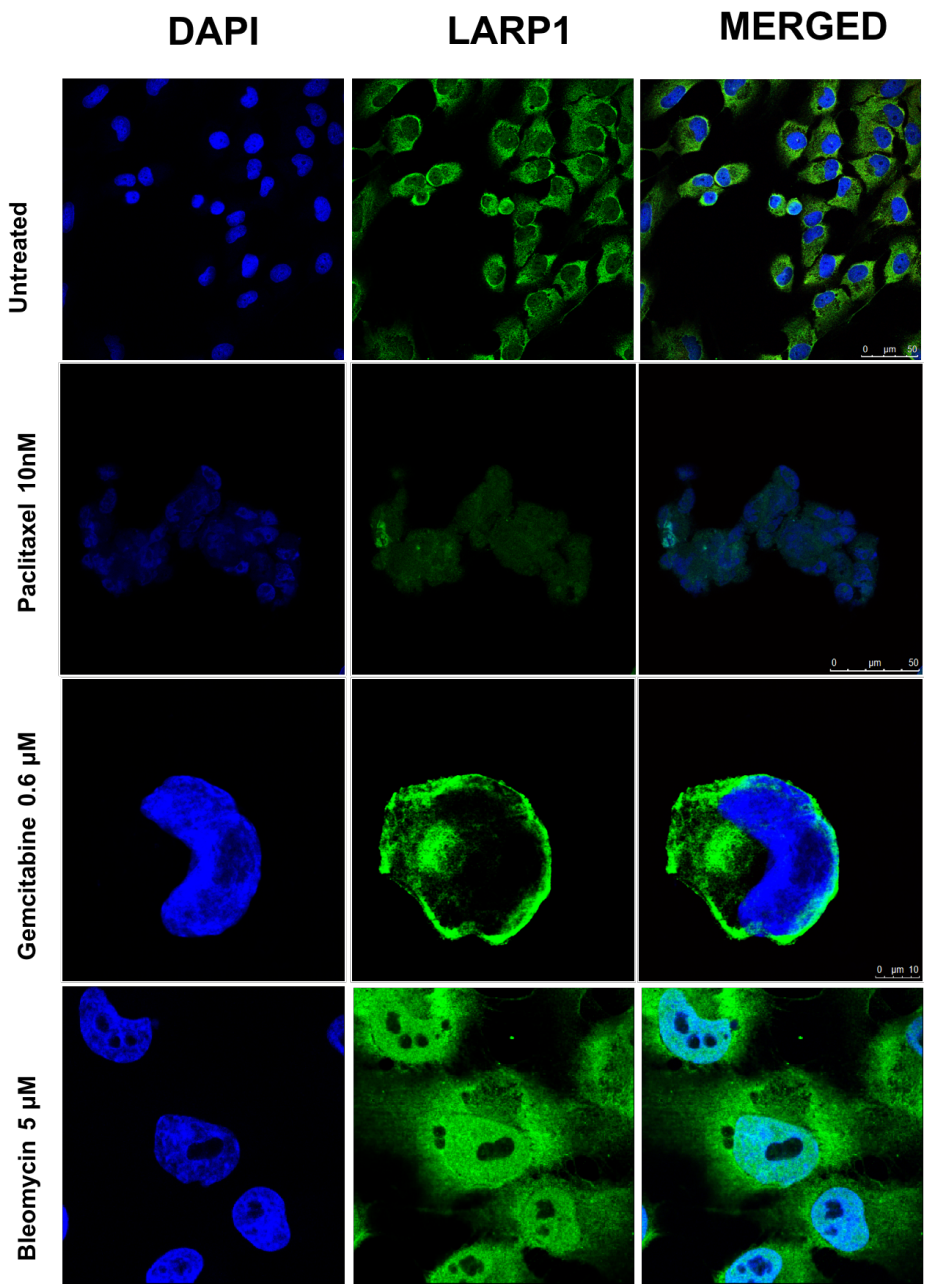


Figure 3-23 LARP1 localisation following treatment with different chemotherapeutic agents in the OVCAR 8 cell line.

Immunofluorescence confocal microscopy of LARP1 (green) with DAPI counterstaining (blue) in OVCAR8 cells. Cells were either untreated or treated with 1) Paclitaxel 10nM for 24h; 2) Gemcitabine 0.6µM for 24h and 3) Bleomycin 5µM over 6h.

3.2.3.2 LARP1 colocalises with PABP1

Both LARP1 and PABP1 are cytoplasmic in the resting OVCAR3 cells but upon treatment with cisplatin (for 24h at a concentration of 10 μM), they colocalise in the nucleus Figure 3-24. Contrary to what is observed for the OVCAR3 cells, in the cisplatin resistant OVCAR8 cell line LARP1 accumulates in the nucleus while PABP1 remains predominantly cytoplasmic after cisplatin treatment Figure 3-25.

PABP1 is known to be diffusely cytoplasmic with the capacity to shuttle between the nucleus and the cytoplasm in response to cellular stress [384]. In HeLa cells it was found to accumulate in the nucleus upon transcription inhibition [359] and UV irradiation [375]. In both cases the nuclear accumulation of PABP1 was governed by the mRNA distribution and a block in mRNA nuclear export. A similar effect is noted in the OVCAR3 cell line but not in the OVCAR8. A speculation could be that the resistant OVCAR8 cells maintain a degree of protein synthesis despite the cisplatin treatment, hence PABP1 maintains its dynamic movement without being restrained in the nucleus. The fact that LARP1 demonstrates a different behaviour in the two cell lines may indicate its versatility and involvement in different cellular functions.

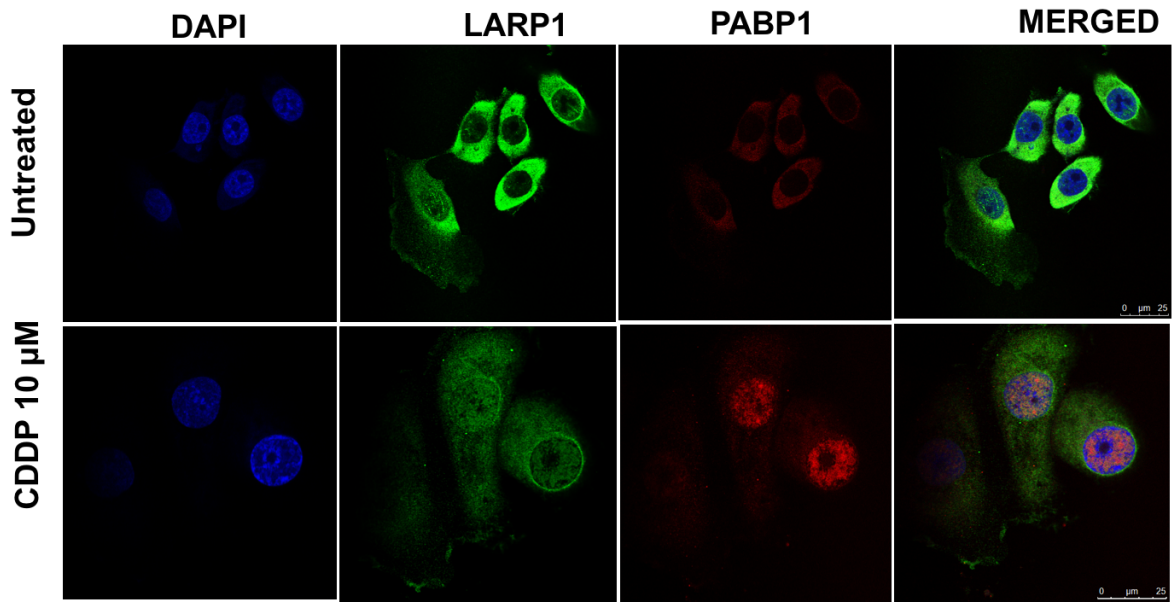


Figure 3-24 LARP1-PABP1 colocalisation in the OVCAR3 cell line.

Immunofluorescence confocal microscopy of LARP1 (green) and PABP1 (red) with DAPI counterstaining (blue) in untreated OVCAR3 cells, or following 24 hours exposure to 10µM CDDP.

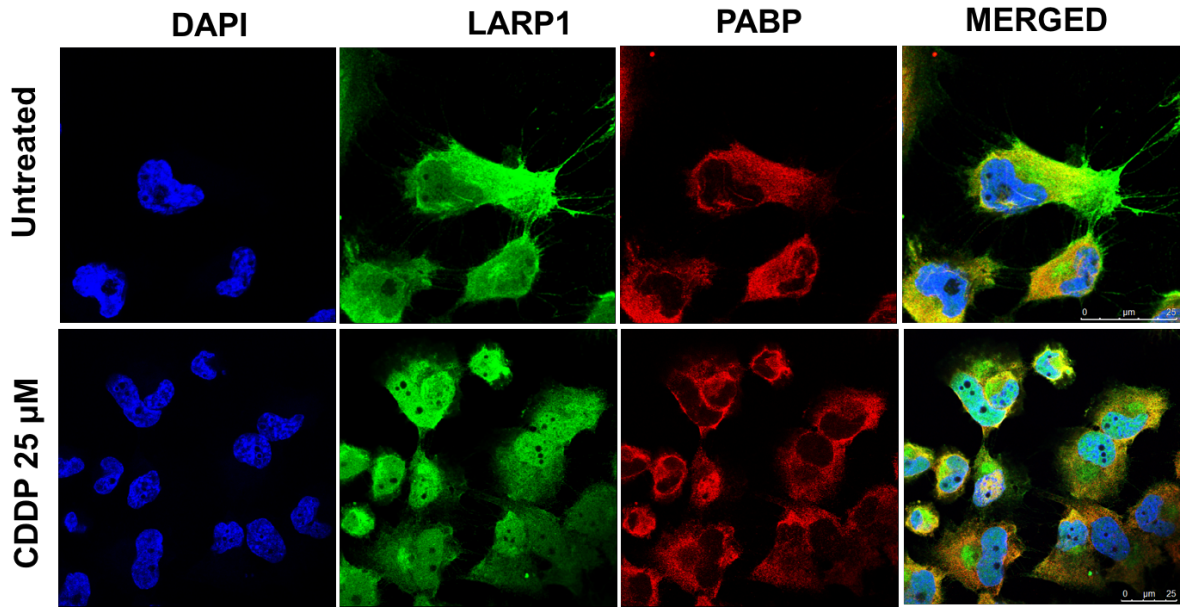


Figure 3-25 LARP1-PABP1 colocalisation in the OVCAR8 cell line.

Immunofluorescence confocal microscopy of LARP1 (green) and PABP1 (red) with DAPI counterstaining (blue) in untreated OVCAR8 cells, or following 24 hours exposure to 25µM CDDP.

3.2.3.3 LARP1 colocalises with PABP4

LARP1 and PABP4 are cytoplasmic in both the untreated OVCAR3 and OVCAR8 cells

Figure 3-26 and Figure 3-27. Treatment of the OVCAR3 cells with cisplatin results in the nuclear colocalisation of LARP1 and PABP4 Figure 3-26. In particular, it seems that they colocalise in granular structures which could possibly represent splicing speckles where poly (A) mRNA accumulates along with a number of proteins involved in pre-mRNA processing [359]. PABP4 is a cytoplasmic protein which, similarly to PABP1, has been previously found to translocate to the nucleus in response to UV irradiation induced genotoxic stress [375]. This relocalisation is suspected to be guided by changes in intracellular distribution of poly(A) RNAs [375].

Contrary to what is observed in the OVCAR3 cell line, in the resistant OVCAR8 cells, cisplatin treatment results in the nuclear accumulation of LARP1, while PABP4 remains cytoplasmic (Figure 3-27). PABP4 seems to follow the same distribution with PABP1in both examined cell lines.

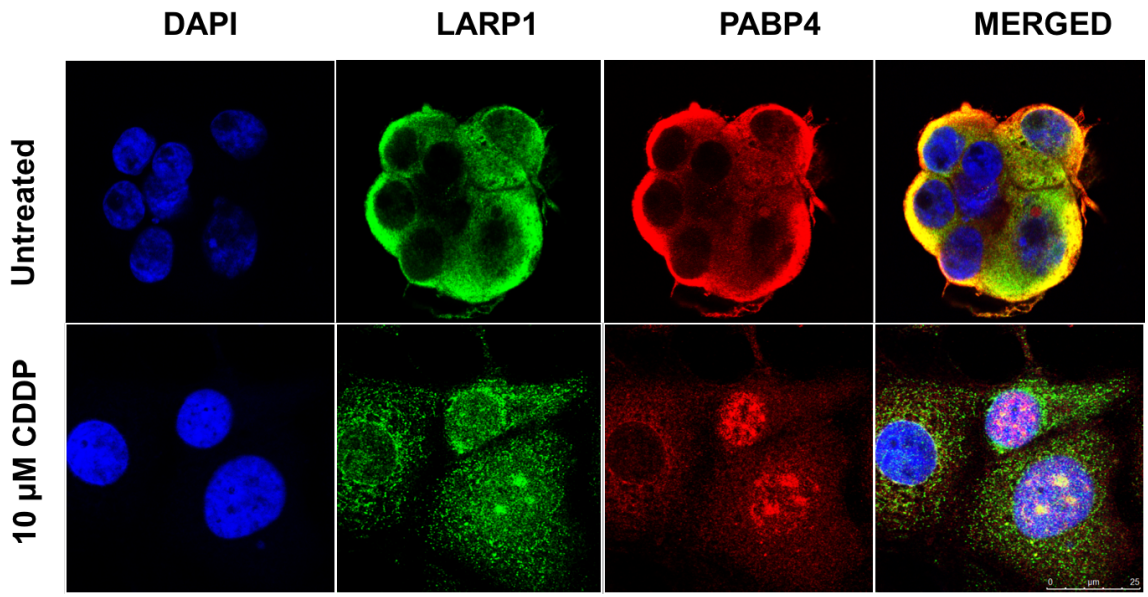


Figure 3-26 LARP1-PABP4 colocalisation in the OVAR3 cell line.

Immunofluorescence confocal microscopy of LARP1 (green) and PABP4 (red) with DAPI counterstaining (blue) in untreated OVCAR3 cells, or following 24 hours exposure to 10µM CDDP.

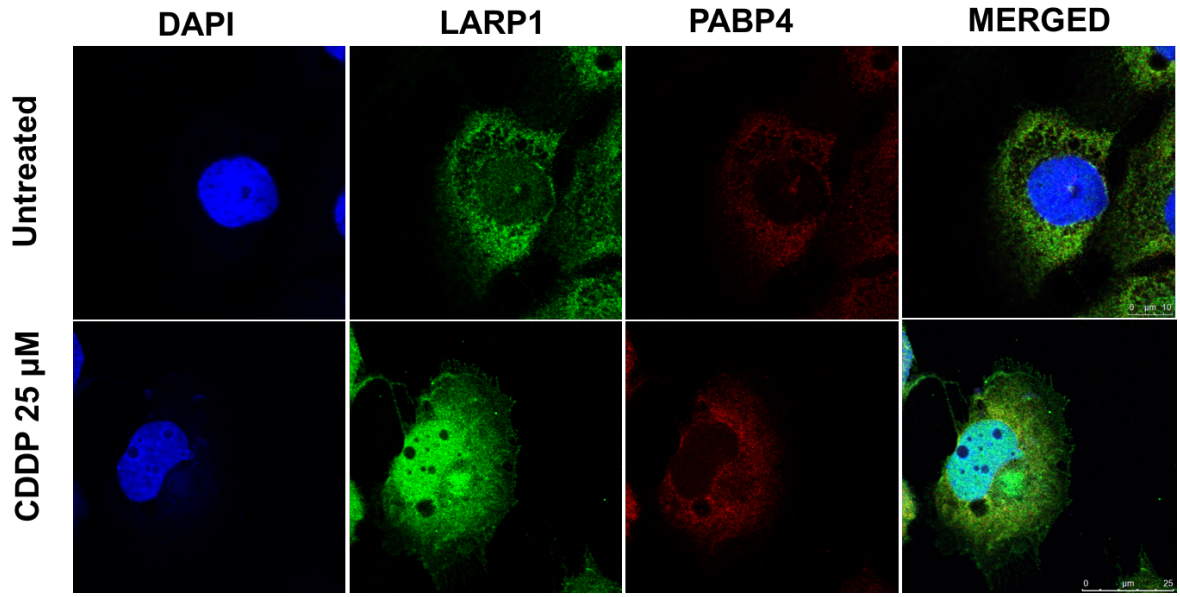


Figure 3-27 LARP1-PABP4 colocalisation in the OVCAR8 cell line.

Immunofluorescence confocal microscopy of LARP1 (green) and PABP4 (red) with DAPI counterstaining (blue) in untreated OVCAR8 cells, or following 24 hours exposure to 25µM CDDP.

3.2.3.4 LARP1 colocalises with YB1

In the OVCAR3 cell line both LARP1 and YB1 proteins colocalise in the cytoplasm of untreated cells. Upon cisplatin treatment, YB-1 colocalises with LARP1 predominantly at the nuclear rim and to a lesser extend in the nucleus (Figure 3-28)

YB1 colocalises with LARP1 in the cytoplasm of OVCAR8 cells at a resting state. Both proteins follow similar patterns of subcellular distribution upon cisplatin treatment and colocalise in the nucleus, the nuclear rim and also in perinuclear cytoplasmic foci (Figure 3-29).

Despite their nuclear localisation upon cisplatin, LARP1 and YB1 maintain a degree of cytoplasmic colocalisaton in both examined cell lines.

YB1 is known to be predominantly localised in the cytoplasm of both malignant and benign cells [385]. It has been reported to translocate to the nucleus in response to a number of stresses including DNA damage [367], viral infection [386] and hyperthermia [387] .

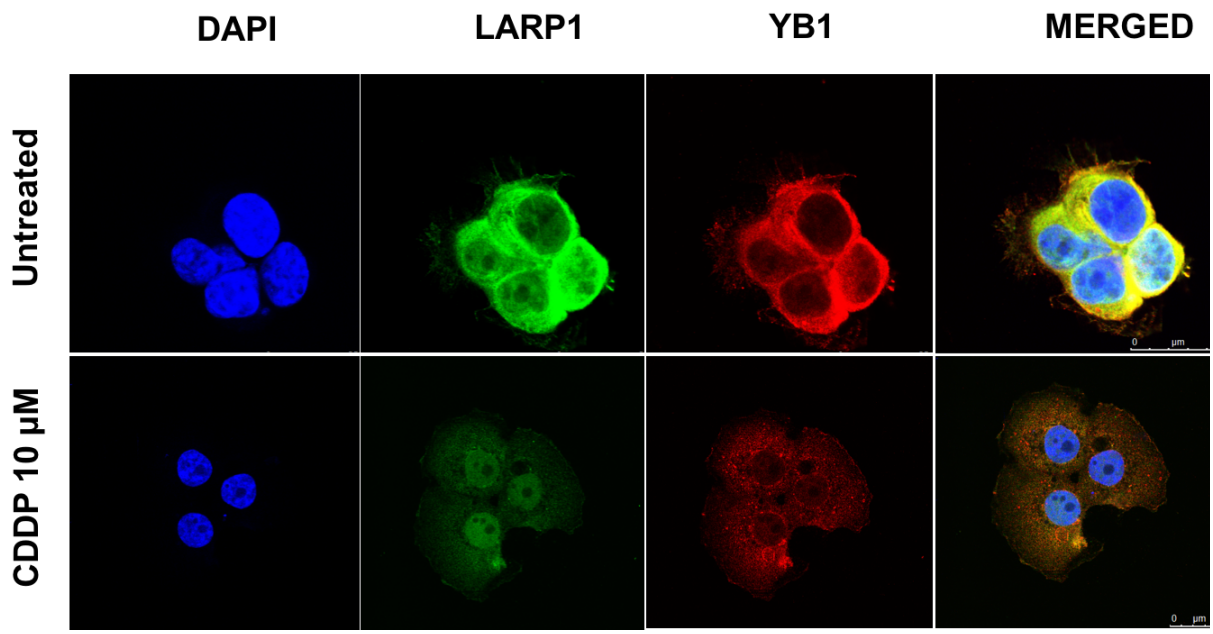


Figure 3-28 LARP1-YB1 colocalisation in the OVCAR3 cell line.

Immunofluorescence confocal microscopy of LARP1 (green) and YB1 (red) with DAPI counterstaining (blue) in untreated OVCAR3 cells, or following 24 hours exposure to 10µM CDDP.

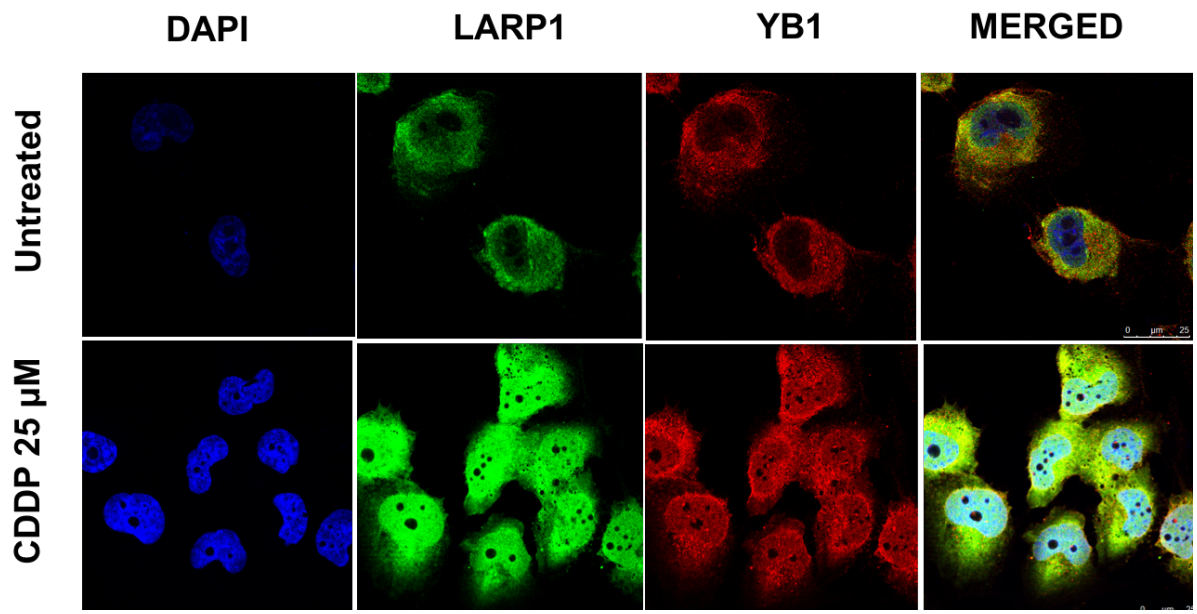


Figure 3-29 LARP1-YB1 colocalisation in the OVCAR8 cell line.

Immunofluorescence confocal microscopy of LARP1 (green) and YB1 (red) with DAPI counterstaining (blue) in untreated OVCAR8 cells, or following 24 hours exposure to 25µM CDDP.

3.2.3.4.1 Summary

In all the investigated cancer cell lines (OVCAR3, OVCAR8, A2780, CP70) LARP1 is predominantly cytoplasmic in untreated cells and becomes nuclear upon treatment with cisplatin. This change in its subcellular localisation is triggered by genotoxic damage. PABP1 and PABP4 colocalise with LARP1 in the cytoplasm of the untreated OVCAR8 and OVCAR3 cells. However, after cisplatin treatment both PABP1 and PABP4 remain cytoplasmic in the OVCAR8 cells but become nuclear in the OVCAR3 cell line. YB1 also colocalises with LARP1 at the cytoplasm of OVCAR3 and OVCAR8 cells at a resting state. Nuclear translocation of YB1 upon cisplatin is evident in OVCAR8 and to a lesser extent in the OVCAR3 cell lines where it colocalises with LARP1 predominantly at the nuclear rim.

3.2.4 CLOSE PROXIMITY INTERACTIONS – DUOLINK PLA ASSAY

To investigate whether LARP1is in close proximity with its identified protein interactors, I used the Proximity Ligation Assay (PLA) - Duolink® [388]. This method is used for the detection, localisation and quantification of interactions of proteins which are in close proximity (<40nm) as described in the methods section.

3.2.4.1 LARP1 is in close proximity with PABP1, PABP4 and YB1

3.2.4.1.1 Interactions between LARP1 and PABP1

Although immunofluorescence studies revealed that LARP1 colocalises with PABP1 in the cytoplasm of untreated OVCAR8 and OVCAR3 cells (section 3.2.3.2), there appear to be only few close interactions between the two proteins as identified by the Duolink-PLA assay Figure 3-30. After treatment with cisplatin, it is evident that LARP1–PABP1 interactions remain quite sparse and predominantly cytoplasmic in OVCAR8 cells, whereas in the OVCAR3 cells they become nuclear. These findings are consistent with what was observed by immunofluorescence.

3.2.4.1.2 Interactions between LARP1 and PABP4

In the resting state of both OVCAR8 and OVCAR3 cell lines, LARP1 was found to closely interact with PABP4. Their interactions were abundant and located in the cytoplasm with only few present in the nucleus Figure 3-31. Following cisplatin treatment, it is evident that their interactions remain predominantly cytoplasmic in the OVCAR8 cells. In the OVCAR3 cells, cisplatin treatment results to an increase in the number of nuclear interactions, but the two proteins also maintain a number of close interactions in the cytoplasm. These findings are consistent with what was observed by immunofluorescence. However, there is inconsistency with the immunoprecipitation outcomes in Figure 3-15 as PABP4 was not

found to interact with LARP1 upon cisplatin in both cell lines. This could imply that these two proteins are in close proximity but not in the same complex.

3.2.4.1.3 Interactions between LARP1 and YB1

In both examined cell lines LARP1 is in close proximity and abundantly interacts with YB1 predominantly in both the cytoplasm and the nucleus. Upon cisplatin treatment in the OVAR8 cells, LARP1 interacts with YB1 only in the nucleus and in the area around the nucleus (Figure 3-32). In the OVCAR3 cells, cisplatin treatment results to fewer interactions between LARP1 and YB1 located in both the nucleus and the cytoplasm.

Again, there is a discrepancy with the immunoprecipitation results in the cisplatin treated OVCAR3 cells (Figure 3-15) which could reflect that LARP1 is in proximity with YB-1 but not in the same ribonucleoprotein complex

To obtain a negative control for the Duolink PLA, I performed the assay with a protein that is known to not bind directly to LARP1. The protein eIF4E was proved to not be a direct interactor of LARP1 and was used as negative control for the experiment [325]. No interaction was identified as expected Figure 3-33.

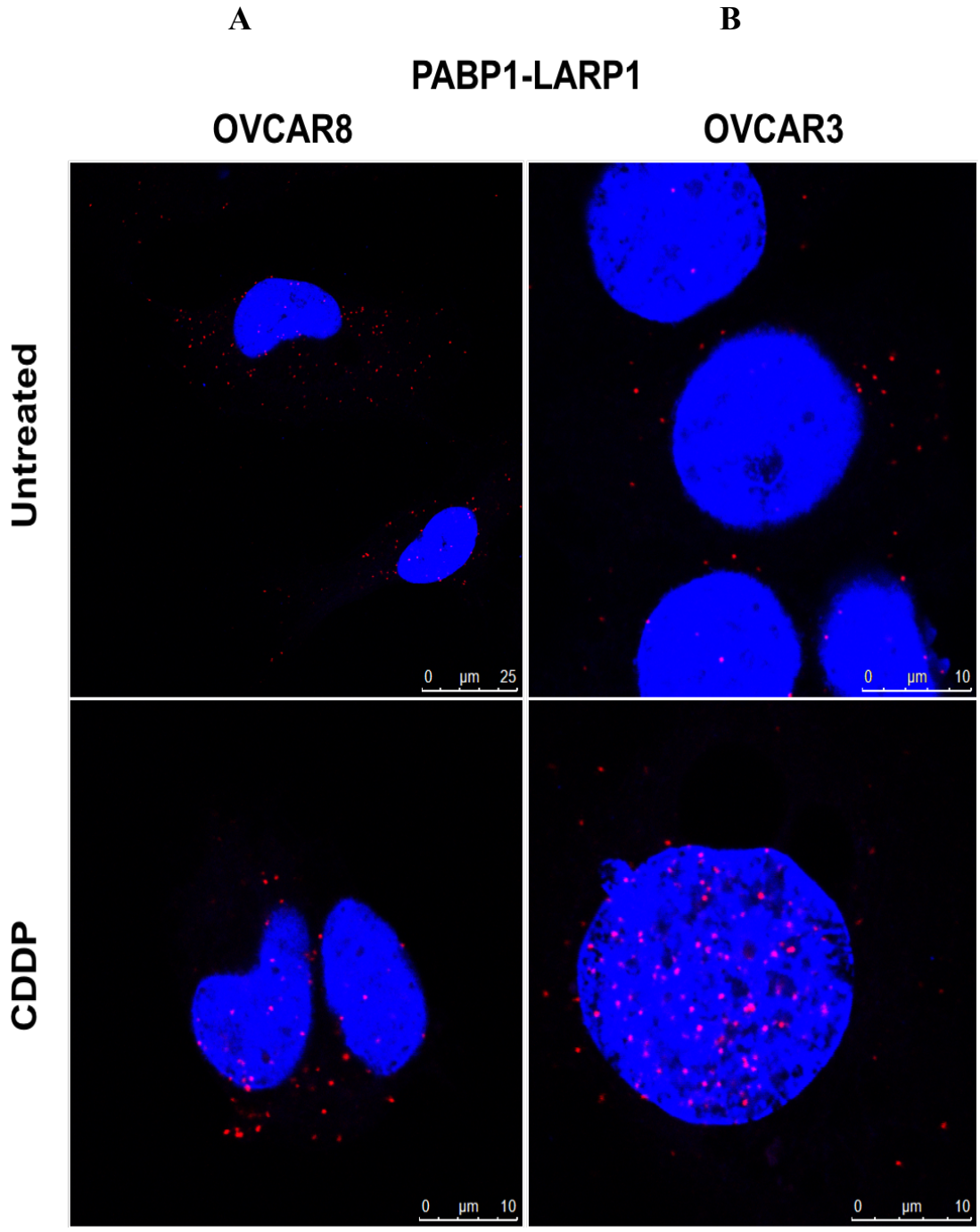


Figure 3-30 Duolink PLA assay for LARP1 interactions with PABP1.

A. Interactions between LARP1and PABP1 in the OVCAR8 cell line before and after treatment with Cisplatin (24 hours at a concentration of 25 µM).
B. Interactions between LARP1and PABP1 in the OVCAR3 cell line before and after treatment with Cisplatin (24 hours at a concentration of 10 µM).
Each spot represents a single interaction

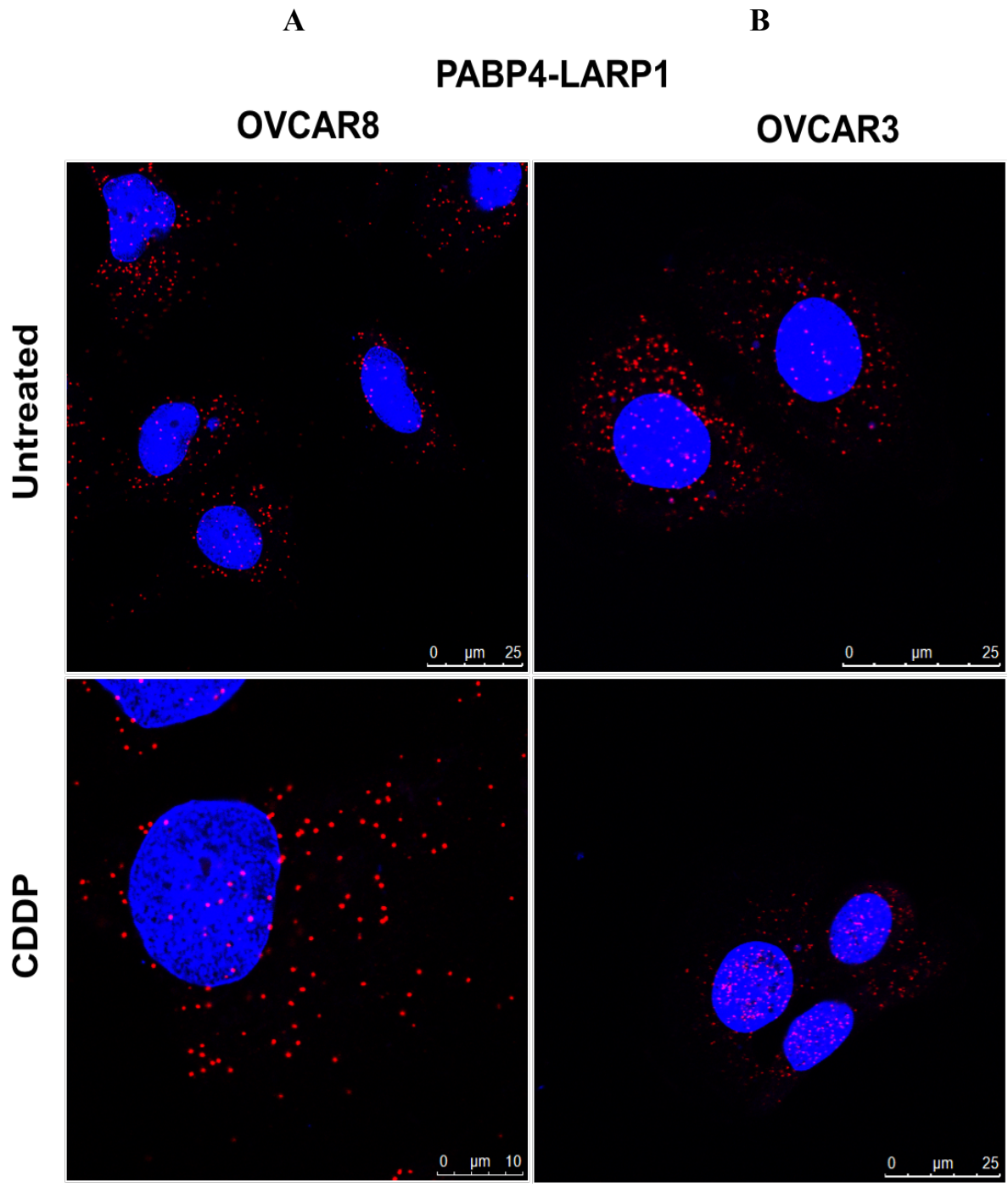


Figure 3-31 Duolink PLA assay for LARP1 interactions with PABP4.

A. Interactions between LARP1and PABP4 in the OVCAR8 cell line before and after treatment with Cisplatin (24 hours at a concentration of 25 µM).
B. Interactions between LARP1and PABP4 in the OVCAR3 cell line before and after treatment with Cisplatin (24 hours at a concentration of 10 µM).
Each spot represents a single interaction

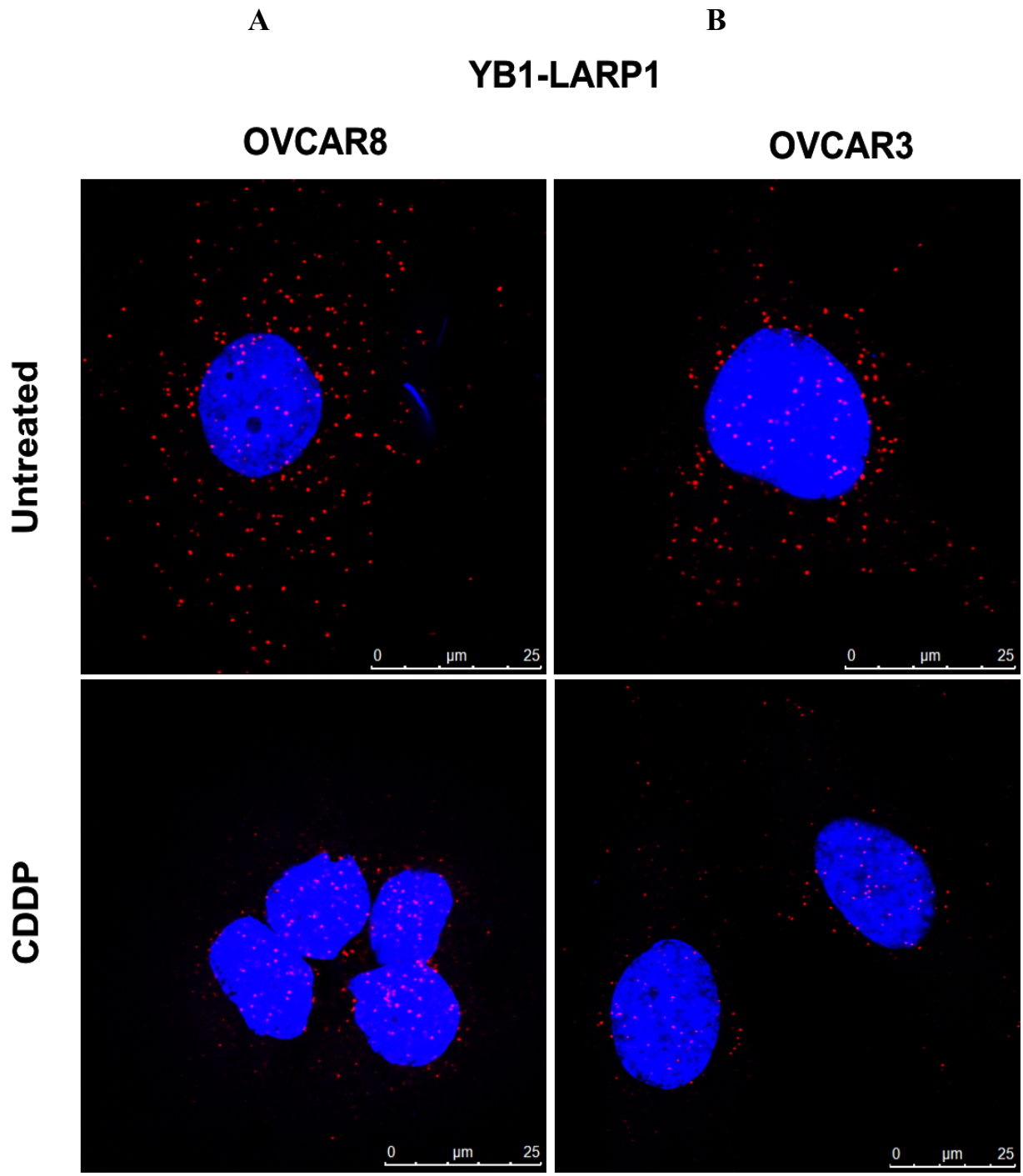


Figure 3-32 Duolink PLA assay for LARP1 interactions with YB1.

A. Interactions between LARP1 and YB1 in the OVCAR8 cell line before and after treatment with Cisplatin (24 hours at a concentration of 25 µM).
B. Interactions between LARP1 and YB1 in the OVCAR3 cell line before and after treatment with Cisplatin (24 hours at a concentration of 10 µM).
Each spot represents a single interaction

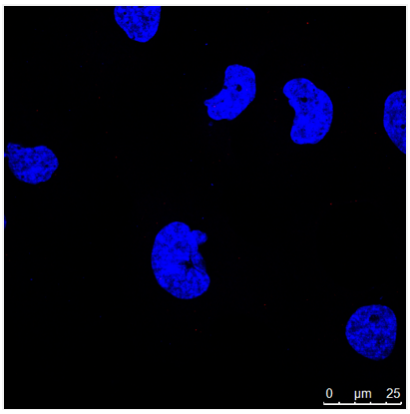


Figure 3-33 Duolink PLA for LARP1 and eIF4E in the OVCAR8 cell line.

The known non-direct interaction between LARP1and eIF4E was used as negative control.

3.2.4.1.4 Summary

In this section, with the use of the proximity ligation assay (PLA) - Duolink®, I demonstrated that LARP1 not only colocalises, but is also in close proximity with PABP1, PABP4 and YB1. At a resting state, these interactions are predominantly cytoplasmic in both OVCAR3 and OVCAR8 cell lines. Upon cisplatin, in the resistant OVCAR8 cell line, the interactions of LARP1 with PABP1 and PABP4 remain cytoplasmic, whereas only the LARP1-YB1 interactions become nuclear. Whereas, in the OVCAR3 cells, the interactions of LARP1 with PABP1 and PABP4 become more nuclear. LARP1-YB1 interactions are markedly decreased and located in both the nucleus and cytoplasm. The subcellular localisation of these interactions is in agreement with what was seen in the immunofluorescence studies and summarised in Table 3-14.

Table 3-14 Subcellular localisation of the interactions between LARP1 and its targets.

Proteins	Cell lines			
	OVCAR8		OVCAR3	
	Untreated	Treated	Untreated	Treated
LAPR1-PABP1	C	C	C	N
LARP1-PABP4	C	C	C	N
LARP1-YB1	C	N	C	C&N

Based on the subcellular compartment where the majority of interactions are seen (C: Cytoplasmic; N: Nuclear)

3.2.5 LARP1 IS REQUIRED FOR MAINTAINING TRANSLATION UPON CISPLATIN TREATMENT

Protein translation is crucial for cell growth and proliferation as well as cell survival in response to stress conditions [188]. Aberrations in the translational control underpin key processes involved in tumorigenesis and can also drive chemotherapy resistance through the selective translation of mRNAs that promote cell survival [188, 389]. Therefore, components of the translational apparatus have been brought to the centre of ongoing research as potential therapeutic targets [208, 334].

The proteomic analyses done in this project as well as evidence from the current literature support a significant involvement of LARP1 in translation [323, 390]. With the use of the surface sensing of translation (SUnSET) assay (kindly performed by Dr Manuela Mura), I investigated the impact of LARP1 on protein synthesis in the presence and absence of cisplatin in both resistant (OVCAR8) and sensitive (OVCAR3) ovarian cancer cells. Cycloheximide and insulin were used as a negative and positive controls respectively.

As seen in Figure 3-34 cisplatin does not significantly affect translation in the resistant OVCAR 8 cell line, whereas an opposite effect is noted in the sensitive OVCAR3 cells in which translation is almost completely abolished. Cisplatin can decrease translation not only via cellular apoptosis but also due to its inhibitory effect on the formation of the initiation complex [391]. The preservation of translation in the cisplatin resistant cells during genotoxic stress could possibly drive their survival via the selective expression of mRNA transcripts such as anti-apoptotic factors. Interestingly, LARP1 knockdown in combination with cisplatin, results in a complete disruption of de novo protein synthesis in both resistant and sensitive cells. Not only this suggests a key role of LARP1 in maintaining translation and consequently promoting cell survival under stress conditions but it further supports its synergistic antitumor action with cisplatin.

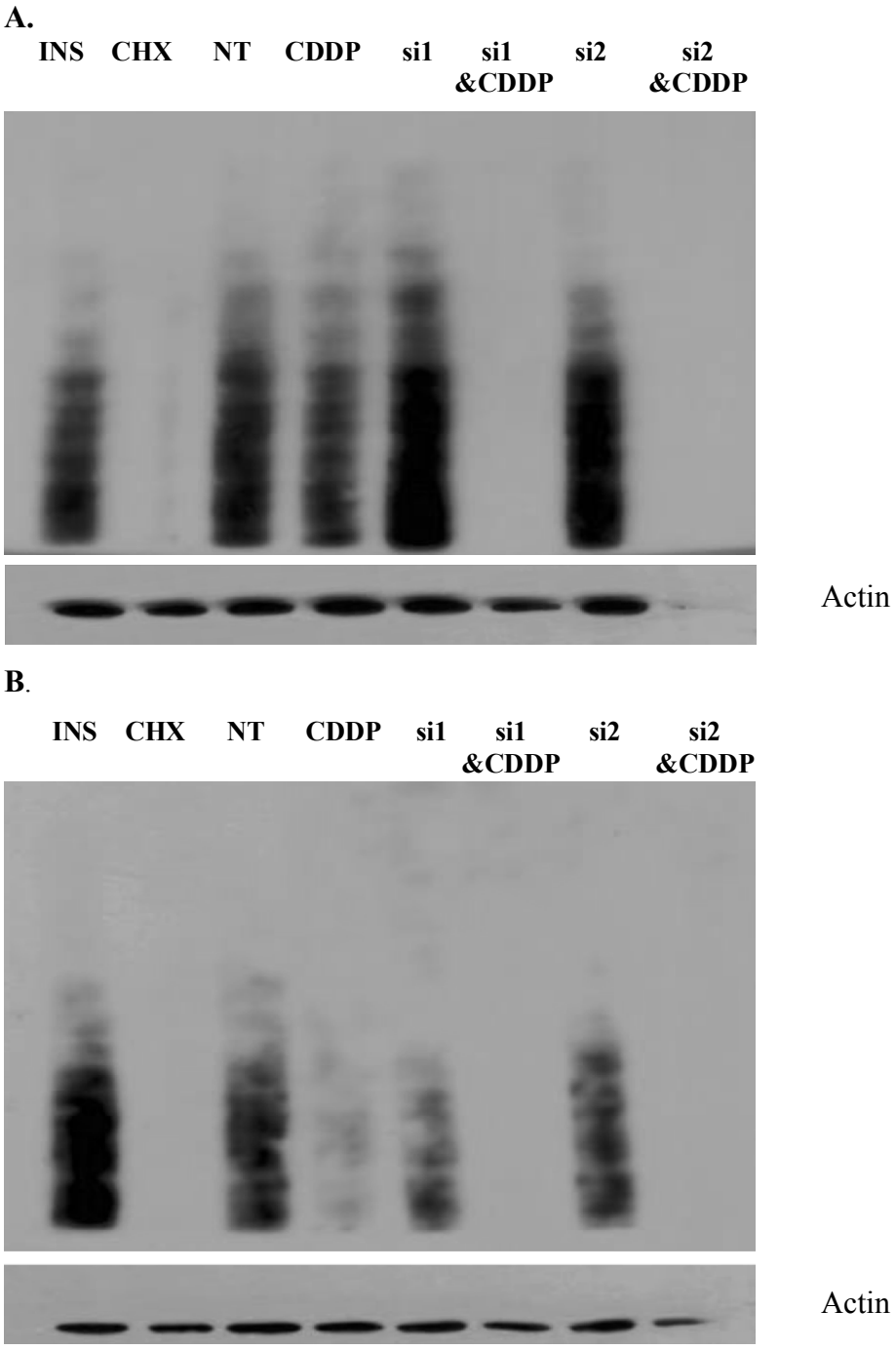


Figure 3-34 LARP1 is required for maintaining translation during genotoxic stress.

Surface sensing of translation (SUnSET) assay showing the effect of LARP1 knockdown with two independent siRNAs on "de novo" translation in the presence and absence of cisplatin in the resistant OVCAR8 (A) and sensitive OVCAR3 (B) cell lines. Insulin and cycloheximide were used to stimulate and inhibit translation respectively as positive and negative controls. Cisplatin treatment disrupted translation in the sensitive OVCAR3 cells, whereas it did not affect it in the resistant OVCAR8 cell line. A combination of LARP1 knockdown and cisplatin resulted in the complete abolishment of protein translation in the resistant cells indicating the key role LARP1 plays in maintaining protein synthesis during genotoxic stress. (INS: insulin, NT: no treatment, CHX: cycloheximide, si1: siLARP1 (1), si2: siLARP1 (2), CDDP: cisplatin) (Experiment performed by Dr Manuela Mura).

3.2.6 THE MAJORITY OF LARP1 PROTEIN INTERACTIONS ARE RNA DEPENDENT

So far, I have identified that PABP1, PABP4 and YB1 are key components of the LARP1 RNP complex and appear to interact with LARP1 in close proximity (<40nm) as shown in section 3.2.4.1. All the above proteins have RNA-binding capacities and LARP1 is known to be in complex with approximately 3000 mRNAs as identified by RNA immunoprecipitation and microarray profiling (RIP-Chip) in HeLa cells [327]. Therefore, I wished to investigate whether the interactions between LARP1 and its identified interactors are mediated by RNA. To achieve this, I immunoprecipitated the LARP1 RNP complex in the OVCAR8 cell line and performed RNA digestion with Micrococcal Nuclease (MNase) as described in methods section (2.4) and summarised in Figure 3-35 (A). MNase degrades both RNA and DNA to 3' phosphomononucleotides and dinucleotides. The eluted samples were analysed by UHPLC-MS/MS mass spectrometry. The identified proteins were cross referenced with those found in complex with LARP1 in the absence of MNase.

In both treated and untreated OVCAR8 cells, LARP1 loses almost 80% of its binding partners upon MNase digestion

Figure 3-35 (B, C). In the untreated cells, LARP1 maintains RNA independent interactions predominantly with ribosomal proteins (Table 3-15). This is not the case for the treated cells as LARP1 maintains interaction mainly with cytoskeletal proteins (Table 3-16). This could imply that, at a resting state, LARP1 is located at the ribosomes and involved in protein translation, which is also supported by the proteomic and functional analyses described in section 3.1. Following treatment with cisplatin, LARP1 changes its direct interactors, maybe due to a change in its functions during genotoxic stress or its nuclear translocation. It is uncertain whether LARP1 plays an active role in this change or it passively follows the redistribution of other proteins in response to genotoxic stress. The RNA independent

interaction of LARP1 with proteins such as annexin and tubulin following cisplatin, may also indicate that they could facilitate its subcellular translocation during genotoxic stress [392]

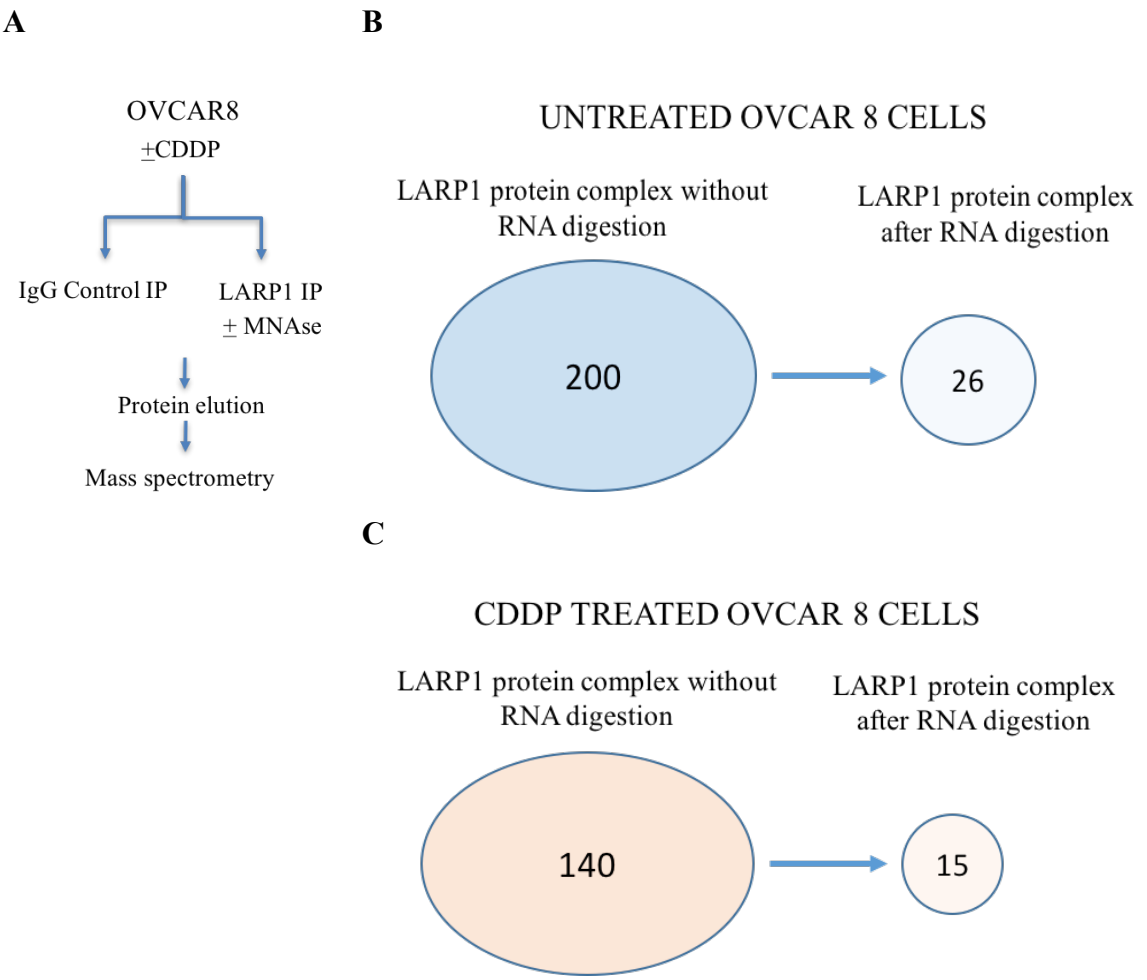


Figure 3-35 LARP1 immunoprecipitation with RNA digestion.

A. Schematic of LARP1 immunoprecipitation coupled with RNA digestion using Micrococcal Nuclease (MNAse)
B. Schematic showing the number of LARP1 immunoprecipitated proteins before and after RNA digestion in the untreated OVCAR8 cells.
C. Schematic showing the number of LARP1 immunoprecipitated proteins before and after RNA digestion in the cisplatin treated OVCAR8 cells (24h at a concentration of 25µM).

Table 3-15 Proteins remaining bound to LARP1 following RNA digestion in the OVCAR8 untreated cells.

Accession No	Protein	Description
P46781	RS9	40S ribosomal protein S9
P62857	RS28	40S ribosomal protein S28
P62258	1433E	14-3-3 protein epsilon
P31944	CASPE	Caspase-14
P25311	ZA2G	Zinc-alpha-2-glycoprotein
Q9HB00	DSC	Desmocolin
F8VPE8	RPLP0	60S acidic ribosomal protein PO
Q96P63	SERPINB12	Serpin B12
Q01804	OTUD4	OTU domain-containing protein 4
P62280	RPS11	40S ribosomal protein S11
A0PJ48	TAOK2	TAOK2 protein
E7ETK0	RPS24	40S ribosomal protein S24
P60866	RPS20	40S ribosomal protein S20
P10599	TXN	Theioredoxin
P62854	RPS26	40S ribosomal protein S26
E5RI99	RPL30	60S ribosomal protein L30
Q5VVC9	RPL11	60S ribosomal protein L11
E9PKZ0	RPL8	60S ribosomal protein L8
F8W0G4	PCBP2	Poly (rC)-binding protein 2
Q8WVV4	POF1B	Protein POF1B
D3YTB1	RPL32	60S ribosomal protein L32
H0YDD8	RPLP2	60S acidic ribosomal protein P2
J3KTJ8	RPL26	60S ribosomal protein L26
P62906	RPL10	60S ribosomal protein L10
K7ENK5	EPG5	Ectopic P granules protein 5 homolog

Table 3-16 Proteins remaining bound to LARP1 following RNA digestion in the cisplatin treated OVCAR8 cells.

Accession No	Protein	Description
P13639	EFE2	Elongation factor 2 OS
P54886	P5CS	Isoform Short of Delta-1-pyrroline-5-carboxylate synthase OS
F5H5D3	TUBA1C	Tubulin alpha-1C chain OS
A0A024R1X8	JUP	Junction plakoglobin, isoform CRA a
Q53HF2		Heat shock 70kDa protein 8 isoform 2 variant
H0YMM1	ANXA2	Annexin
Q14204	DYNC1H1	Cytoplasmic dynein 1 heavy chain 1
B4DUR8	CCT3	T-complex protein 1 subunit gamma
P12273	PIP	Prolactin-inducible protein
P10599	TXN	Thioredoxin
Q86YZ3	HRNR	Hornerin
Q5VXJ5	SYCP1	Synaptonemal complex protein 1
Q53T40	FHL2	Putative uncharacterized protein FHL2
A0A0S2Z3L4	CTSD	Cathepsin D isoform 2
E7ER08	CYP26B1	Cytochrome P450 26B1

As expected for an RNA-binding protein, these results demonstrate that the vast majority of LARP1 protein interactions in both untreated and cisplatin treated OVCAR8 cells are mediated by RNA. The interactions of LARP1 with its key interactors PABP1, PABP4 and YB1 were also lost after RNA digestion and this was further validated by western blotting Figure 3-36.

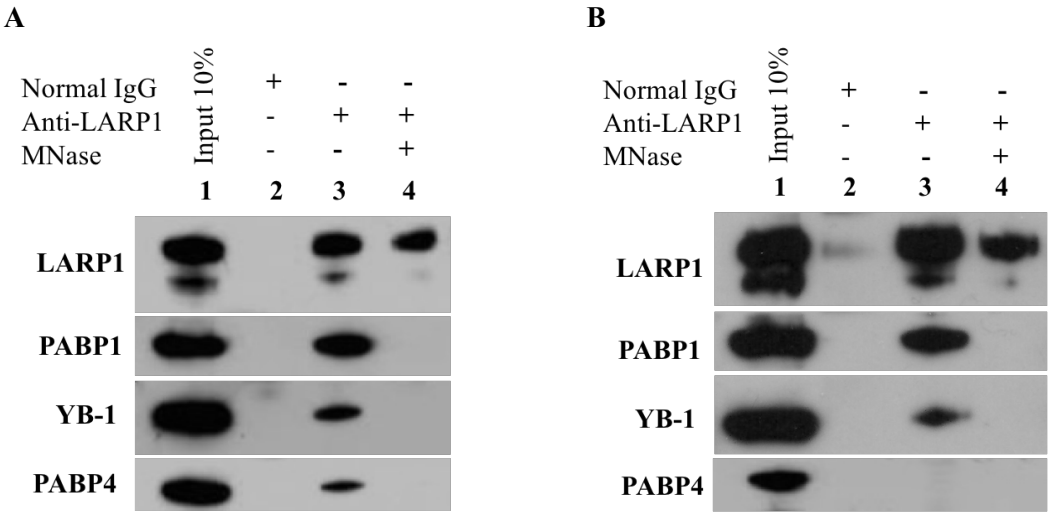


Figure 3-36 Western blot confirming the RNA dependent interaction between LARP1 and its targets.

Immunoprecipitation of LARP1 RNP complex was performed with anti-LARP1 antibody using IgG as negative control in untreated **(A)** and cisplatin treated **(B)** OVCAR8 cells. Part of the immunoprecipitated sample was treated with Micrococcal Nuclease (MNase) to digest all RNA sequences. The immunoprecipitated proteins were identified by western blotting using antibodies against PABP1, PABP4 and YB1. Lane 1: Input (10% whole cell lysate); Lane 2: IgG control; Lane 3: anti-LARP1 antibody; Lane 4: anti-LARP1 antibody + MNase treatment.

3.2.6.1 YB-1 and PABP1 interact via RNA

Since both YB-1 and PABP1 are well recognised mRNA regulators [393-395], I wondered whether their interaction was also RNA-dependent. To answer this, I repeated the previously described methodology and performed YB-1 immunoprecipitation with and without RNA digestion, analysing the precipitates for the presence of PABP1 by western blot. The same process was followed for PABP1. Both proteins were found to interact with each other through RNA

Figure 3-37 which shows that LARP1, PABP1, and YB1 proteins are in complex by sharing common mRNA targets.

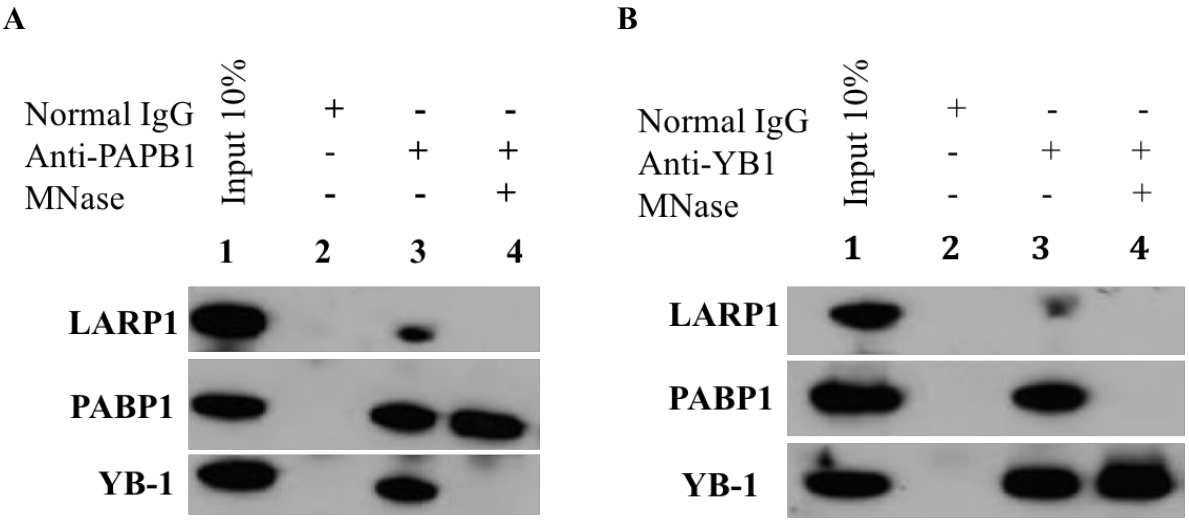


Figure 3-37 Western blot confirming the RNA dependent interaction between PABP1 and YB1.

A. Immunoprecipitation of PABP1 RNP complex was performed with anti-PABP1 antibody using IgG as control in the OVCAR8 cell line. Part of the immunoprecipitated sample was treated with Micrococcal Nuclease (MNase) to digest RNA. The precipitates were analysed for the presence of YB-1 and LARP1 by western blotting. (RNP: Ribonucleoprotein complex)

B. Immunoprecipitation of YB1 RNP complex was performed with anti-YB1 antibody using IgG as control in the OVCAR8 cell line. Part of the immunoprecipitated sample was treated with Micrococcal Nuclease (MNase) to digest RNA. The precipitates were analysed for PABP1 and LARP1 by western blotting.

3.2.7 SUMMARY

In this sub-chapter I validated the mass spectrometry findings by reverse immunoprecipitations and western blotting, confirming the presence of PABP1, PABP4 and YB-1 as key components of LARP1 RNP in both the untreated OVCAR8 and OVCAR3 cell lines. Following cisplatin treatment, the LARP1-PABP1-YB1 complex remains intact in the OVCAR8 cell line whereas in the OVCAR3 cells it gets disrupted with LARP1 losing interaction with YB1 and PABP4. With the use of immunofluorescence, I identified that LARP1 is profoundly cytoplasmic in the resting cells and becomes nuclear upon cisplatin treatment, a pattern which was observed in both cisplatin resistant and sensitive ovarian cancer cell lines. Despite the fact that LARP1 colocalises with PABP1, PABP4 and YB1 in the cytoplasm of untreated cells (OVCAR8 and OVCAR3) their localisation after cisplatin treatment varies in the two cell lines. Only YB-1 was found to follow a similar localisation pattern with LARP in both examined cell lines. Using the Duolink-PLA assay I proved that LARP1 is in close proximity with PABP1, PABP4 and YB1 but their interactions are mediated via mRNA as the complex was disassembled following RNA digestion. This indicates that these proteins share common mRNA targets.

3.3 IDENTIFYING COMMON LARP1-YB1 TRANSCRIPT TARGETS

3.3.1 YB1 IS A KEY LARP1 INTERACTOR

LARP1 and YB1 expression is increased in various cancer cells and they play a significant role in conferring cisplatin resistance, whilst their depletion increases sensitivity to cisplatin [332, 396]. YB-1 overexpression has been associated with cisplatin resistance in ovarian, breast, melanoma and bladder cancer cell lines [369, 397, 398]. Similarly to LARP1, YB1 translocates to the nucleus following DNA-damage [367, 399]. Furthermore, its nuclear localisation correlates with drug resistance and worse survival outcomes [385]. Both LARP1 and YB1 are RNA-binding proteins known to be involved in the post-transcriptional regulation of mRNA transcripts affecting the stability of their mRNA targets [327, 332, 400] and promoting the expression of pro-survival genes such as BCL2 [332, 398, 401, 402].

So far, I have shown that YB1 is a key component of LARP1 complex and these two proteins interact on an RNA dependent manner. Taking this into account, I wished to investigate the common mRNA targets that are part of their RNA-protein complex as this could unveil a potential common mechanism in cisplatin resistance.

3.3.2 COMPARATIVE ANALYSIS OF RNA-SEQUENCING DATABASES UPON LARP1 AND YB1 KNOCKDOWN

I obtained and cross-referenced databases of mRNA transcripts regulated by YB-1 and LARP1 in cisplatin resistant ovarian cancer cell lines. The first database included mRNA-sequencing data showing the differential expression of mRNA transcripts following YB-1 knockdown by siRNA in SKOV3 cells , published by Basaki et al. [403]. The second database contained respective mRNA-sequencing data following LARP1- knockdown by siRNA in OVCAR8 cells, published by Hopkins et al [332]. Cross-referencing these two databases led to the identification of those mRNA transcripts whose abundance is regulated

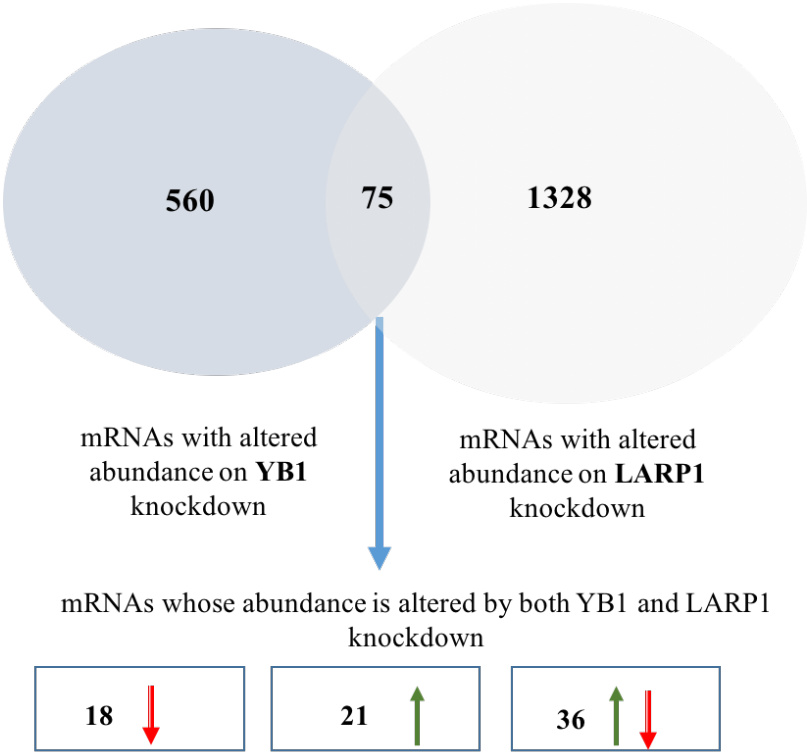
by both proteins. Only mRNAs with altered expression in both YB1 and LARP1 knockdowns were selected. My analysis identified 75 mRNA transcripts whose abundance was affected (decreased or increased) by both YB1 and LARP1 individual knockdowns. Of these, 18 exhibited decreased abundance upon YB1 and LARP1 knockdowns; 21 were increased and 36 were differentially regulated by the two proteins (either decreased on YB1 knockdown and increased on LARP1 knockdown or vice versa) Figure 3-38 (A). I focused only on the mRNA targets whose abundance was similarly affected by LARP1 and YB1 Table 3-17. Functional enrichment analysis for these transcripts revealed that the mRNAs decreased upon LARP1 and YB1 knockdowns were involved in biological processes related to detoxification of organic compounds and response to toxic substance Figure 3-38 (B).

Table 3-17 mRNAs with altered abundance upon LARP1 and YB1 individual knockdowns in cisplatin resistant ovarian cancer cell lines.

mRNAs with decreased abundance		mRNAs with increased abundance	
MMP24	matrix metallopeptidase	GTSE1	G-2 and S-phase expressed 1
RRAGD	Ras related GTP binding	PEX13	peroxisomal biogenesis factor 13
SLC7A11	solute carrier family 7 member 11	RANGAP1	Ran GTPase activating protein 1
PHLDA1	pleckstrin homology like domain family A	DHFR	dihydrofolate reductase
SCAMP1	secretory carrier membrane protein	SLC7A5	solute carrier family 7 member 5
SLC9A1	solute carrier family 30 member 9A1	TUBB4	tubulin, beta 4A
GRB14	growth facror receptor bound protein	ASF1B	ASF1 anti-silencing function 1 homolog
PLOD2	procollagen lysine	CHAF1A	chromatin assembly factor 1
RDH10	retinol dehydrogenase	DDB2	damage-specific DNA binding protein 2
MGAT4A	mannosyl alpha glycoprotein	CDCA8	cell division cycle associated 8
NADSYN1	NAD synthetase	MT1X	metallothionein 1X
DUSP5	dual specificity phospatase	CDC6	cell division cycle 6 homolog
VMP1	vacuole membrane protein	MCM10	minichromosome maintenance complex component 10
WDR1	WD repeat domain	ANLN	anillin
ARHGAP18	Rho GTPAse activating protein	SH3BP4	SH3-domain binding protein 4;
ATP7A	ATPase copper transporting alpha	UNG	uracil-DNA glycosylase
IFI16	interferon gamma inducible protein	ZNF367	zinc finger protein 367
SLC30A1	solute carrier family 30 member 1	UHRF1	UHRF1 binding protein
ATP7B	ATPase copper transporting beta	NID2	nidogen 2
		HMGB2	high mobility group box2
		FBN1	fibrillin 1

Data acquired by cross-referencing published databases [332, 403].

A



B

Functional enrichment analysis

mRNAs with decreased abundance		mRNAs with increased abundance	
Biological Process (GO)	**FDR**	**Biological Process (GO)**	**FDR**
Detoxification of organic compound	0.0269	Mitotic cell cycle	0.00698
Stress response to metal ion	0.0269	Cell cycle	0.00984
Response to toxic substance	0.0347	Mitotic cell cycle process	0.0169

Figure 3-38 YB1 and LARP1 regulate common mRNA targets.

A. Venn Diagram showing the overlap between genes with altered mRNA abundance on YB1 knockdown and LARP1 knockdown. The overlapping genes were sub classified to those with decreased mRNA abundance following both LARP1 and YB1 individual knockdowns; those with increased abundance and those which either decreased upon one protein's knockdown and increased upon the other's or vice versa.

B. Functional enrichment analysis for the genes whose mRNA abundance was similarly affected by the individual knockdowns of LARP1 and YB1.
FDR: False discovery rate.

As seen in Table 3-17, following either LARP1 or YB1 knockdown there were reduced mRNA levels of genes encoding for the copper transporters *ATP7A, ATP7B* and the zinc transporter *SLC30A1* all of which are known to be upregulated in cisplatin resistant ovarian cancer lines and control the cellular intake of cisplatin [404, 405] . On the other hand, there was increased expression of the damaged DNA binding protein gene (*DDB2*), whose overexpression has been reported to enhance sensitivity of ovarian cancer cells to cisplatin [406]. The fact that both LARP1 and YB1 upregulate the expression of genes promoting cisplatin resistance such as *ATP7A, ATP7B* and *SLC30A1* while downregulating ones that enhance cisplatin sensitivity *(DDB2*) was of particular interest and I wished to explore this further.

3.3.3 LARP1 AND YB1 ARE IN COMPLEX WITH TRANSCRIPTS INVOLVED IN CISPLATIN RESISTANCE

Having identified potential common mRNA targets of LARP1 and YB1, I questioned whether these transcripts are directly regulated by LARP1 and YB1 as components of their RNP complexes.

To confirm that the *ATP7A, ATP7B, SLC30A1* and *DDB2* transcripts interacted with both LARP1 and YB1 proteins, I performed RNA immunoprecipitation for LARP1 and YB1 in the OVCAR8 cell line before and after treatment with cisplatin for 24 hours at a concentration of 25 μM (Figure 3-39 A). In the untreated cells *ATP7B, SCL30A1* and *DDB2* were highly enriched in both the anti-LARP1 (Figure 3-39 C) and anti-YB1 immunoprecipitates (Figure 3-40) compared to the *28S* control, confirming that each associated with both LARP1 and YB1. *ATP7A* enriched only in the anti-YB1 immunoprecipitate. As a positive control for the experiment I used *BCL2* whose transcript is a known direct target of LARP1 [332], whereas *28S* ribosomal RNA was used as a negative control.

All the examined targets were more enriched in the anti-YB1 immunoprecipitate (Figure 3-40) when compared to the anti-LARP1 one (Figure 3-39 C). This could be due to a number of factors. Despite the fact that both LARP1 and YB1 proteins are rather abundant in the cell lysates (Figure 3-39 B), there may be a difference in their immunoprecipitated concentrations due to antibody affinity which could lead to greater mRNA abundance in the YB1 immunoprecipitate. Another possibility is that perhaps YB1 has greater affinity for those targets compared to LARP1 in this cell line. It could also be speculated that YB-1 binds its mRNA targets on a less ephemeral manner compared to LARP1.

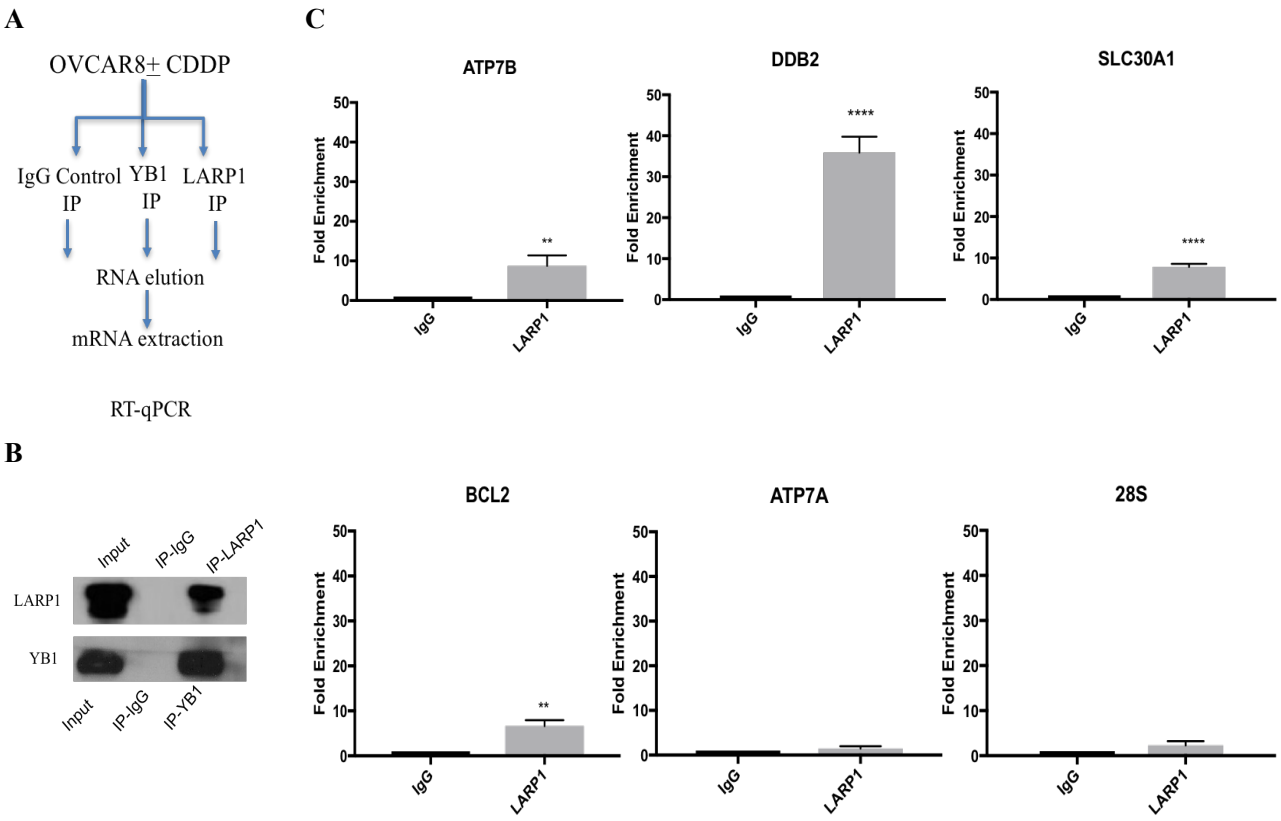


Figure 3-39 mRNA components of the LARP1 RNP complexes in untreated OVCAR8 cells.

A. Schematic of LARP1 and YB1 RNA-immunoprecipitation (RIP) in OVCAR8 cells.

B. Western blot of LARP1 protein following LARP1-immunoprecipitation and YB-1 protein following YB-1 immunoprecipitation in OVCAR8 cells. (Input =10% of the whole cell lysate).

C. Fold enrichment of mRNA transcripts in LARP1 / Isotype control RIP analysed by RT-qPCR (ΔCt). 28S ribosomal protein included as negative control and BCL2 as positive control. Student t-test. Eight experimental repeats. Error bars represent SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

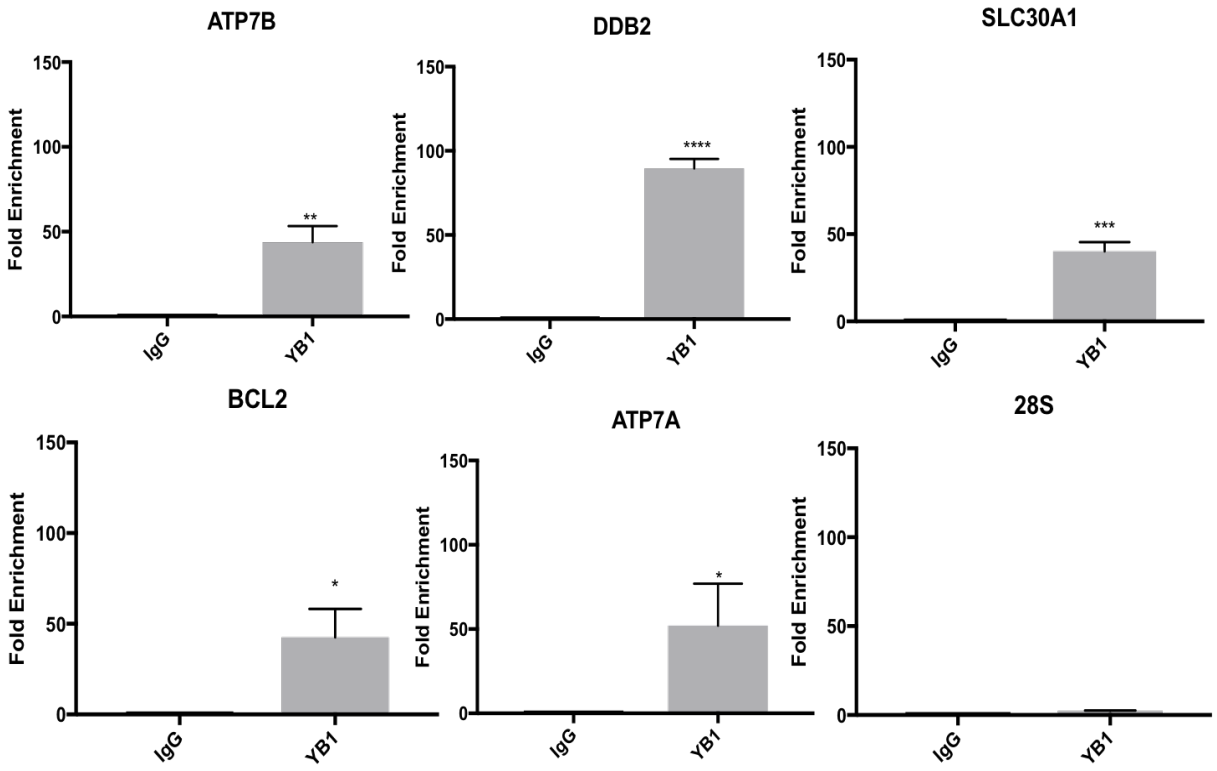


Figure 3-40 mRNA components of the YB1 RNP complexes in the untreated OVCAR8 cells.

Fold enrichment of mRNA transcripts in YB1 / Isotype control RIP analysed by RT-qPCR (ΔCt). 28S ribosomal protein included as negative control and BCL2 as positive control. Student t-test. Eight experimental repeats. Error bars represent SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Following cisplatin treatment, *ATP7B, DDB2, SLC30A1* and *BCL2* transcripts remained complexed with both LARP1 (Figure 3-41) and YB1 (Figure 3-42). ATP7A which was found as a component of the YB1 complex in the resting OVACR8 cells, did not enrich in the cisplatin treated cells (data not shown). SLC30A1 was more enriched in both the LARP1 and YB1 immunoprecipitates upon cisplatin, and DDB2 increased its enrichment only in the LARP1 immunoprecipitate. All the other targets remained unchanged. The preservation of the LARP-YB1 RNP complex following cisplatin treatment could potentially be an indicator of its significance in mediating cisplatin resistance in the OVCAR 8 cell line.

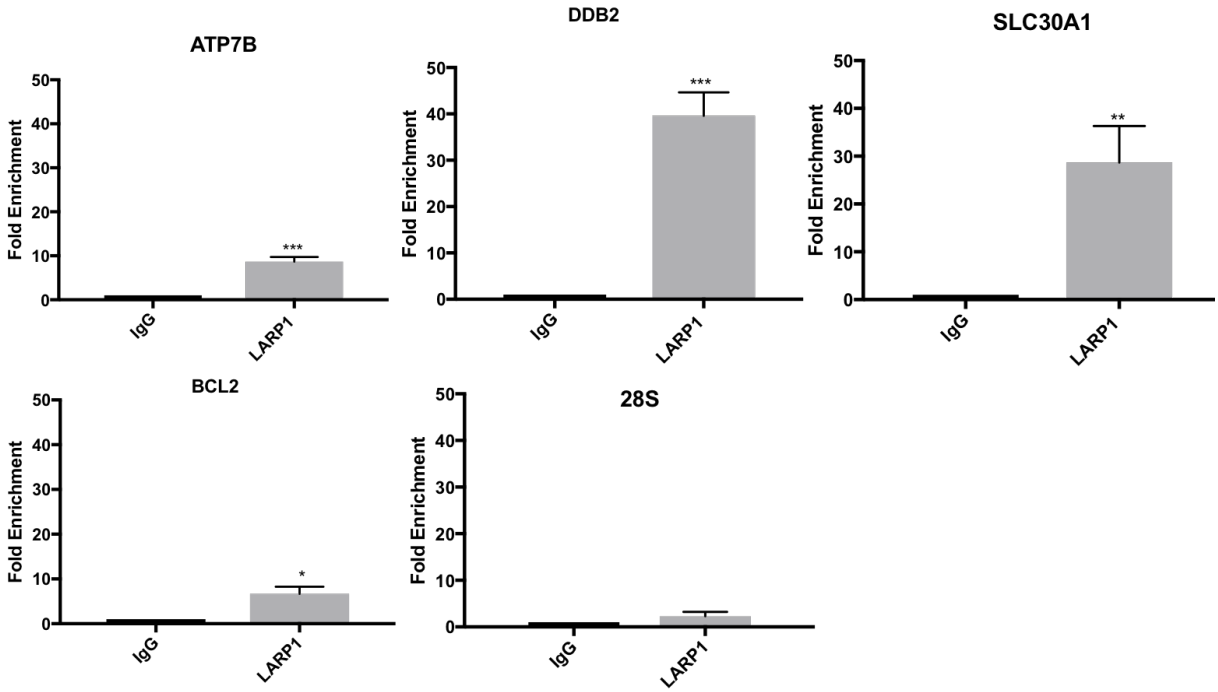


Figure 3-41 mRNA components of the LARP1 RNP complex in cisplatin treated OVCAR8 cells.

Fold enrichment of mRNA transcripts in LARP1 / Isotype control RIP analysed by RT-qPCR (ΔCt). 28S ribosomal protein included as negative control and BCL2 as positive control. Student t-test. Eight experimental repeats. Error bars represent SEM. (* p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

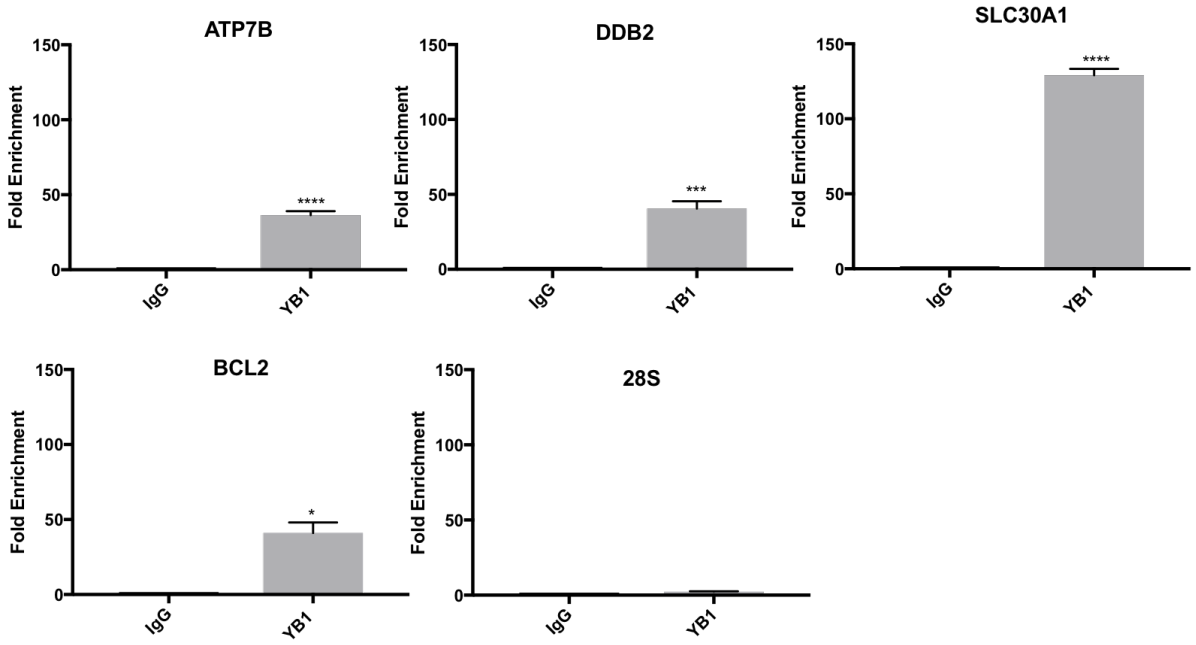


Figure 3-42 mRNA components of the YB1 RNP complex in cisplatin treated OVCAR8 cells.

Fold enrichment of mRNA transcripts in YB1 / Isotype control RIP analysed by RT-qPCR (ΔCt). 28S ribosomal protein included as negative control and BCL2 as positive control. Student t-test. Eight experimental repeats. Error bars represent SEM. (* p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.3.4 LARP1 AND YB1 CO-REGULATE THE EXPRESSION OF GENES INVOLVED IN CISPLATIN RESISTANCE

Having established that *ATP7B, SLC30A1* and *DDB2* are targets of both LARP1 and YB1, I wished to validate the previously identified effect these have on their abundance. I repeated the LARP1 and YB1 knockdowns using two independent siRNAs for each and measured the mRNA levels of the ATP7B, SLC30A1 and DDB2 genes by RT-qPCR. My results confirmed the previously described RNA-seq data for LARP1 knockdown Figure 3-43 (A) showing a decrease in the abundance of ATP7B and SLC30A1 and an increase in DDB2. However, they were partly consistent with the RNA-seq data for YB1 knockdown. I found that YB1 knockdown resulted to a decrease in ATP7B and an increase in the mRNA levels of both *SLC30A1* and *DDB2* the investigated targets Figure 3-43 (B). A reason for this discrepancy in the abundance of *SLC30A1* could be the fact that the RNA-seq data were obtained in the cisplatin resistant ovarian cancer SKOV3 cell line but my validation was done in the OVCAR8 cell line which I have used so far in this project as a model of cisplatin resistance.

My validation confirms that LARP1 and YB1 co-regulate at the mRNA level the expression of genes that are involved in cisplatin resistance such as *ATP7B, SLC30A1* and *DDB2.* Both proteins are found to upregulate the expression of the cisplatin efflux pump *ATP7B* [125] while downregulating the expression of the pro-apoptotic factor *DDB2* [406, 407].

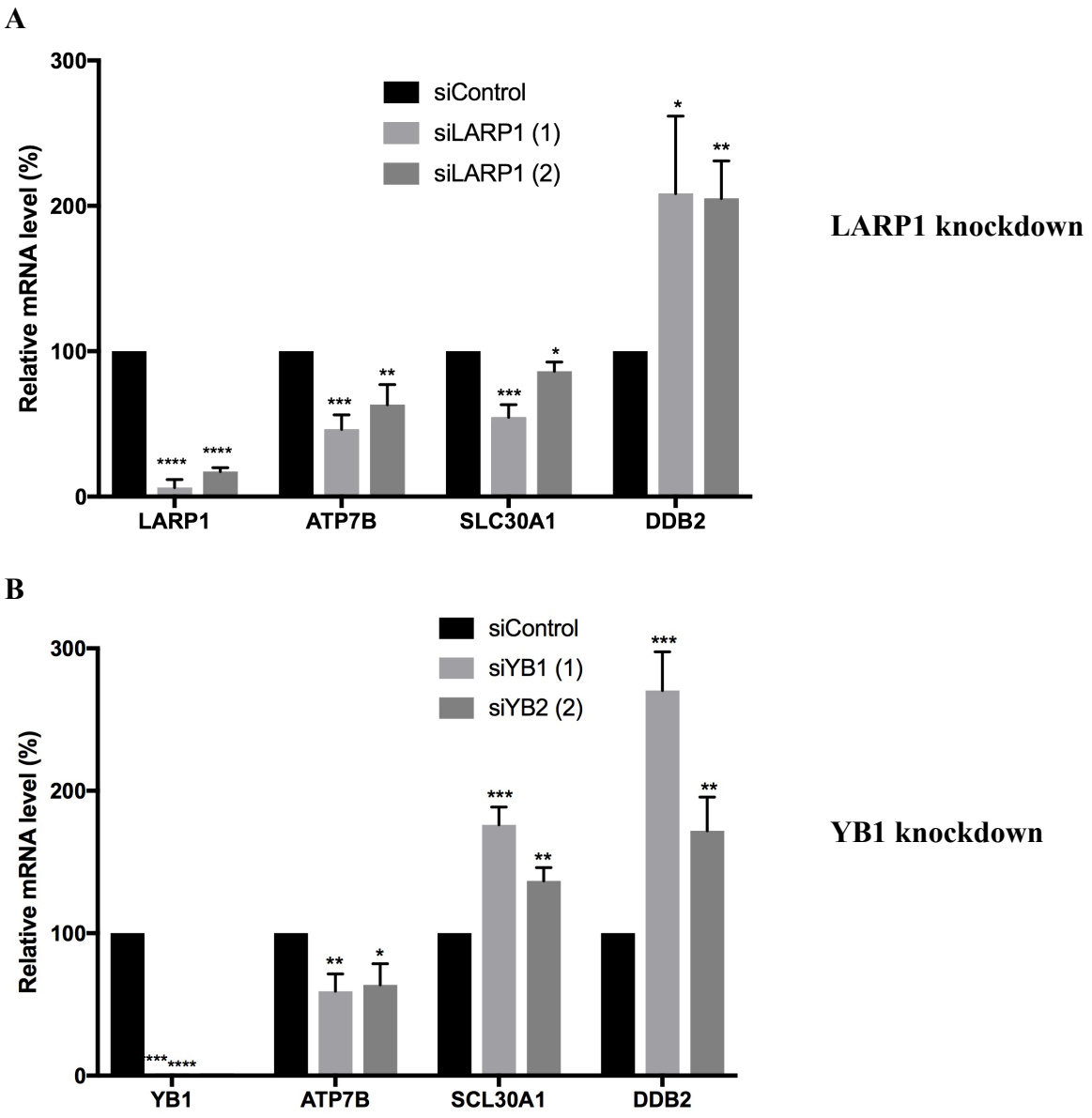


Figure 3-43 RNA-seq data validation for the identified targets in the OVCAR8 cell line.

A. RT-qPCR analysis of percentage change in mRNA levels of putative LARP1 targets upon LARP1 knockdown with two independent siRNAs.

B. RT-qPCR analysis of percentage change in mRNA levels of putative LARP1 targets upon LARP1 knockdown with two independent siRNAs.

(ΔΔCt, normalised to 18S RNA). Student t-test ****p<0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Minimum of three experimental repeats. Error bars represent SEM.

3.3.5 LARP1 AND YB1 CO - REGULATE THE EXPRESSION OF THE CISPLATIN RESISTANCE GENE ATP7B AT RNA AND PROTEIN LEVEL

ATP7B is a copper (Cu) transporter, member of the P-type ATPase transporters family, and primarily involved in facilitating coper efflux from the cell [408]. *ATP7B* has been found to be overexpressed at both mRNA and protein levels in a number of human cancers including ovarian, breast, gastric, hepatocellular, oral squamous cell and prostate cancer [409-413]. Increased expression of *ATP7B* has been directly associated with cisplatin resistance in various cell lines, by detoxifying the cells and decreasing intracellular cisplatin accumulation [125, 410, 414]. In cisplatin resistant ovarian cancer cell lines, *ATP7B* has been identified as a potential therapeutic target and its siRNA-mediated depletion has a synergistic effect with cisplatin leading to increased apoptosis [129]. Furthermore, *ATP7B* expression holds a predictive value in ovarian cancer and its overexpression has been correlated with unfavourable clinical outcomes in ovarian cancer patients receiving cisplatin-based chemotherapy [415].

ATP7B plays a key role in conferring cisplatin resistance and its mRNA is complexed with both LARP1 and YB-1 which exert similar effects on its abundance, upregulating its expression. As seen in Figure 3-43 and Figure 3-44 (A), individual knockdowns of LARP1 and YB-1 result in a decrease in ATP7B mRNA levels with LARP1 knockdown having a greater effect compared to YB-1. I speculated that a double knockdown of LARP1 and YB-1 could have an even greater effect on the ATP7B mRNA abundance. However, as seen in Figure 3-44 (A), double knockdown was not as effective as the individual knockdowns in reducing the ATP7B mRNA. To ensure this was not due to inadequate siRNA knockdown I repeated the experiment using a combination of LARP1 shRNA and YB-1 siRNA (data not shown) obtaining the same trends. Apart from showing no synergy between LARP1 and YB-1 concurrent knockdown in reducing the abundance of ATP7B mRNA, this also demonstrates

that a double knockdown is less effective than single LARP1 knockdown. A similar effect was also noted at a protein level with LARP1 knockdown resulting to almost a 35% decrease in ATP7B protein levels, YB-1 knockdown to a 15% decrease and a double knockdown to approximately 20% decrease respectively (Figure 3-44 B, C). In view of this observation, I then investigated the possibility of a regulatory loop being present between LARP1 and YB-1. As will be further explained in section 3.3.6, LARP1 and YB-1 regulate each other and YB-1 knockdown results in an increase in LARP1. This could potentially explain why a double YB-1/LARP1 knockdown is not as effective as single LARP1 knockdown in reducing the mRNA abundance of ATP7B.

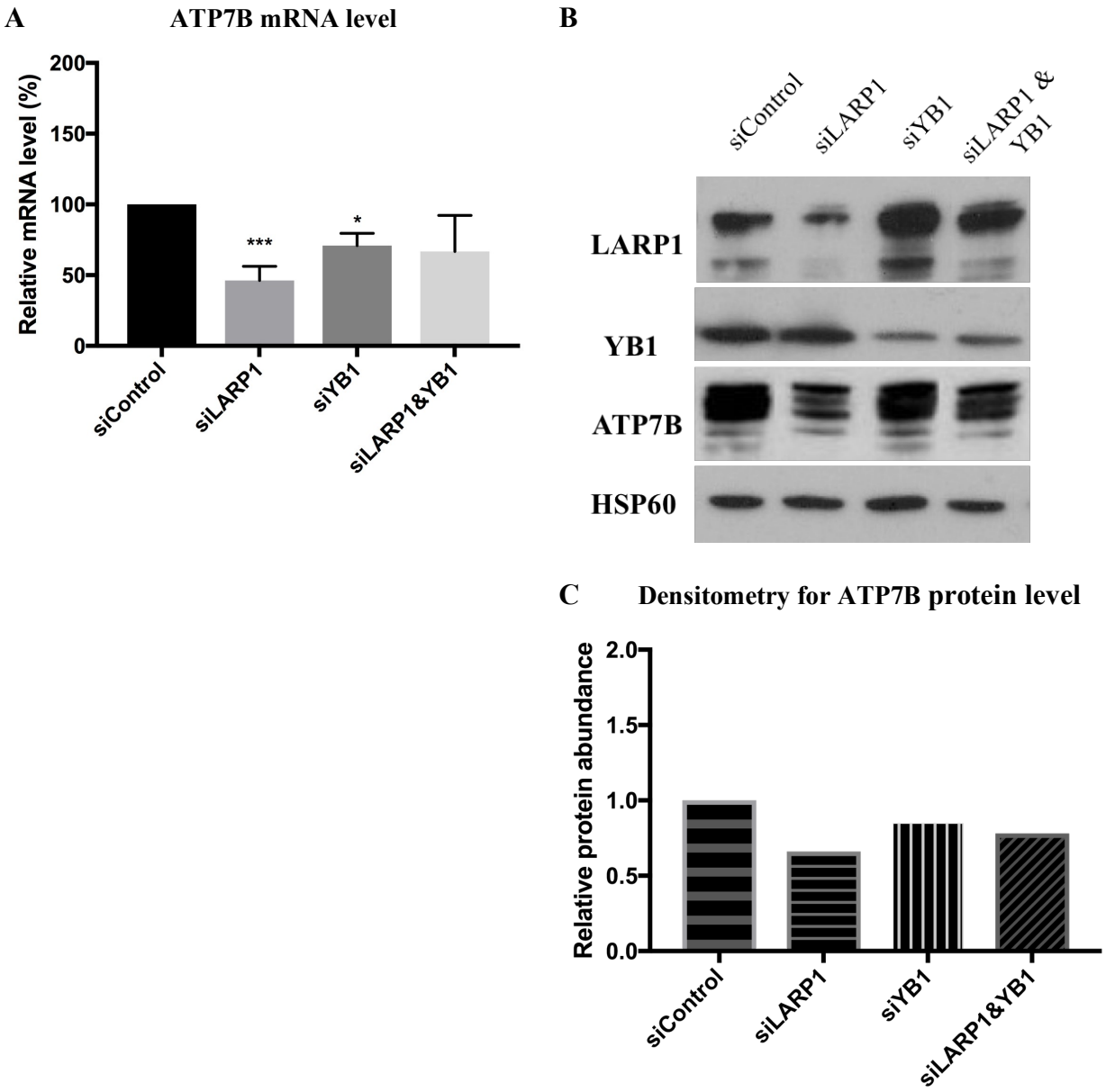


Figure 3-44 LARP1 and YB1 regulate the abundance of ATP7B at an mRNA and protein level.

A. RT-qPCR analysis of percentage change in mRNA levels of ATP7B following LARP1, YB-1 individual knockdowns and double LARP1/YB-1 knockdown. (ΔΔCt, normalised to 18S RNA). ***P < 0.001, **P < 0.01, *P < 0.05. Student t-test. Minimum of three experimental repeats.
Error bars represent SEM

B. Western blot analysis of ATP7B protein levels following LARP1 and YB-1 individual knockdowns and double LARP1/YB-1 knockdown. (Blot adjusted from Figure 3-46).

C. ATP7B protein levels were further quantified by densitometry.

3.3.6 LARP1 AND YB-1 RECIPROCALLY REGULATE THEIR OWN TRANSCRIPTS

While investigating the effect of LARP1 and YB-1 on their common targets ATP7B, it became evident that YB-1 knockdown increased the protein level of LARP1 Figure 3-44(B), which could indicate that YB-1 regulates LARP1 expression. To identify a possible effect of YB-1 on LARP1 mRNA levels and vice versa, I knocked down each one using two different siRNAs and quantified their mRNA levels with RT-qPCR. YB-1 knockdown led to an increase in LARP1 mRNA levels Figure 3-45 (A), whereas LARP1 knockdown decreased the mRNA levels of YB-1Figure 3-45 (B). This indicates that both proteins exert an opposite regulatory effect on each other, with LARP1 having a positive regulation on YB-1 and YB-1 having a negative one on LARP1 at an mRNA level. Double YB-1 and LARP-1 knockdown led to a decrease in the levels of both transcripts. However, the obtained decrease was not as effective as compared to that achieved with each one's individual knockdown Figure 3-45. As also mentioned in section 3.3.5, to ensure this was not a technical error of the siRNA knockdown, I repeated the double knockdown using a combination of LARP1 shRNA and YB siRNA (data not shown) obtaining the same outcome. Besides this reciprocal regulatory effect on mRNA abundance, which could be partially responsible for the observed outcome in combination knockdown experiments, YB-1 and LARP1 are both important mRNA regulators and their simultaneous decrease may activate other compensatory mechanisms to sustain their mRNA levels.

At a protein level, YB-1 knockdown increased LARP1 abundance by 50% (Figure 3-46 A, B) which was consistent with the changes noted at the mRNA level Figure 3-45 (B). On the contrary, LARP1 knockdown did not alter the protein abundance of YB-1Figure 3-46 (C), despite decreasing its mRNA levels Figure 3-45 (B). This could be attributed to the half–life of YB-1 protein. As the knockdown in this experiment was sustained for only 48 hours, a lengthier knockdown would be useful to further study this trend.

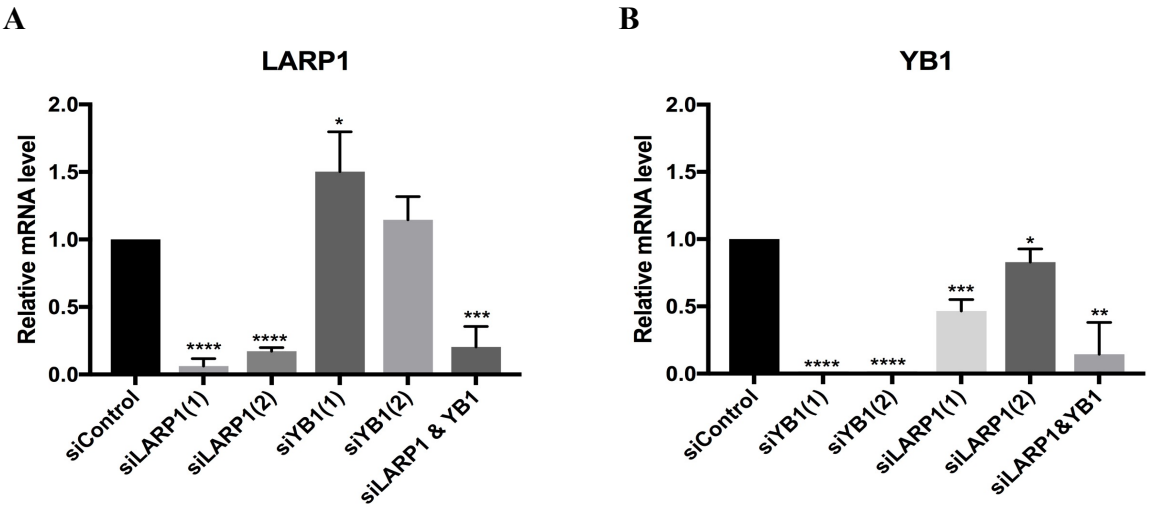


Figure 3-45 LARP1 and YB-1 reciprocally regulate each other's mRNA abundance.

A. RT-qPCR analysis of percentage change in mRNA levels of LARP1 following LARP1, YB-1 individual knockdowns and double LARP1/YB-1 knockdown.

B. RT-qPCR analysis of percentage change in mRNA levels of YB-1 following LARP1, YB-1 individual knockdowns and double LARP1/YB-1 knockdown.
(ΔΔCt, normalised to 18S RNA). ***P < 0.001, **P < 0.01, *P < 0.05. Student t-test. Minimum of three experimental repeats. Error bars represent SEM

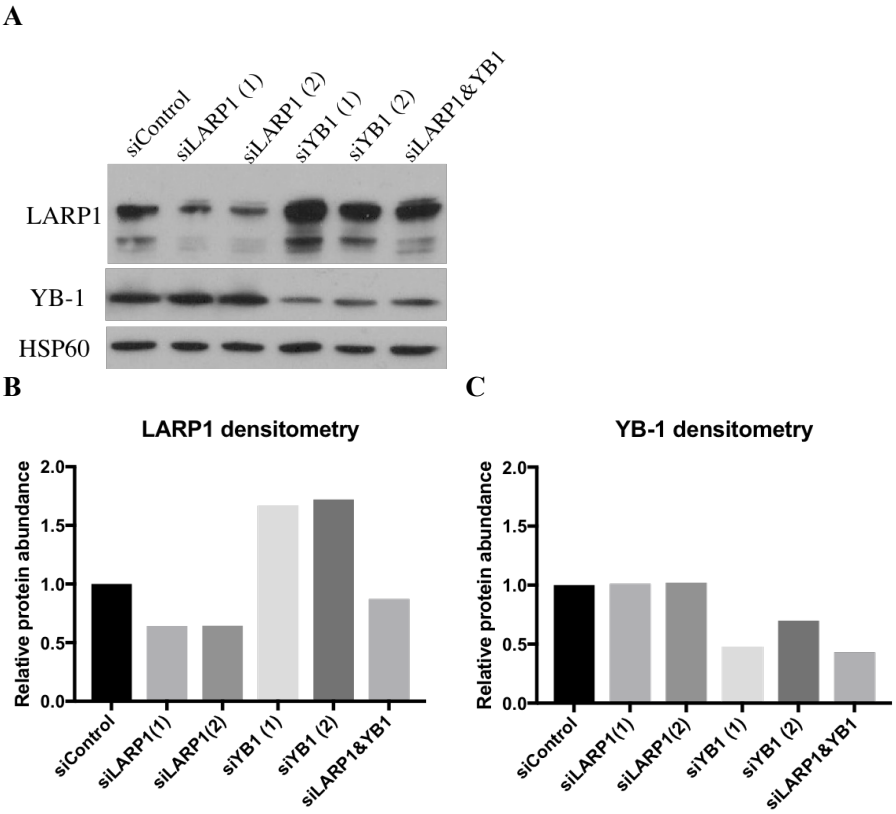


Figure 3-46 YB-1 affects LARP1 abundance at a protein level.

A. Western blot analysis of YB-1 and LARP1 protein levels following their own individual knockdowns and double LARP1/YB-1 knockdown.
B. Densitometry analysis for LARP1 protein levels; **C.** Densitometry analysis for YB-1 protein levels.

Since both YB-1 and LARP1 regulate each other's expression at the mRNA and protein level, I suspected that they may be in complex with each other's mRNA. To investigate this, I performed RNA immunoprecipitation for LARP1 and YB1 in the OVCAR8 cell line. Only YB-1 mRNA was significantly enriched in the anti-LARP1 immunoprecipitate compared to 28S control. Whereas, both YB-1 and LARP1 mRNAs were enriched in the anti-YB-1 immunoprecipitate Figure 3-47. YB-1 has been known to bind its own mRNA [416] which was also confirmed by my findings.

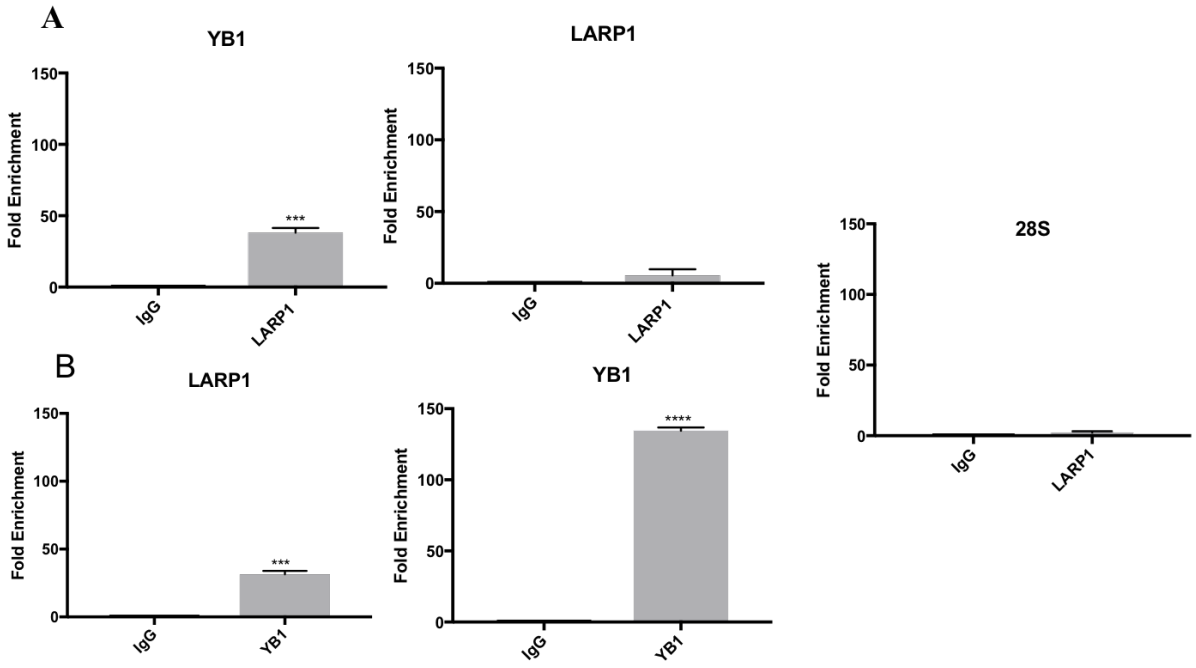


Figure 3-47 LARP1 and YB-1 are in complex with their own mRNA transcripts.

Fold enrichment of transcripts in LARP1 / IgG control RIP **(A)** and YB-1/ IgG control RIP **(B)**

Analysed by RT-qPCR (ΔCt). 28S ribosomal protein included as negative control. Student t-test. Minimum of 3 experimental repeats. Error bars represent SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

3.3.7 LARP1 REDUCES CISPLATIN –DNA ADDUCT FORMATION

One of the key mechanisms underpinning cisplatin resistance is the decrease of intracellular cisplatin concentration by the cancer cells leading to less genotoxic damage [94]. This is mainly mediated by the overexpression of copper efflux pumps, and particularly ATP7B which, as seen in section 3.3.5, is regulated by the LARP1- YB1 complex with LARP1 having a greater impact on its expression [129]. To further test this potential mechanism, I used inductively coupled plasma (ICP) – mass spectrometry to measure the amount of DNA-bound cisplatin before and after LARP1 knockdown in OVCAR8 cells treated with cisplatin at a concentration of 25μM for 5 hours (Figure 3-48). This showed that LARP1 knockdown increased the amount of DNA-bound cisplatin by almost 2 times. This further supports a potential mechanism in which LARP1 plays a key role in promoting cisplatin resistance via the post-transcriptional regulation of the ATP7B transcript as a component of its mRNPs.

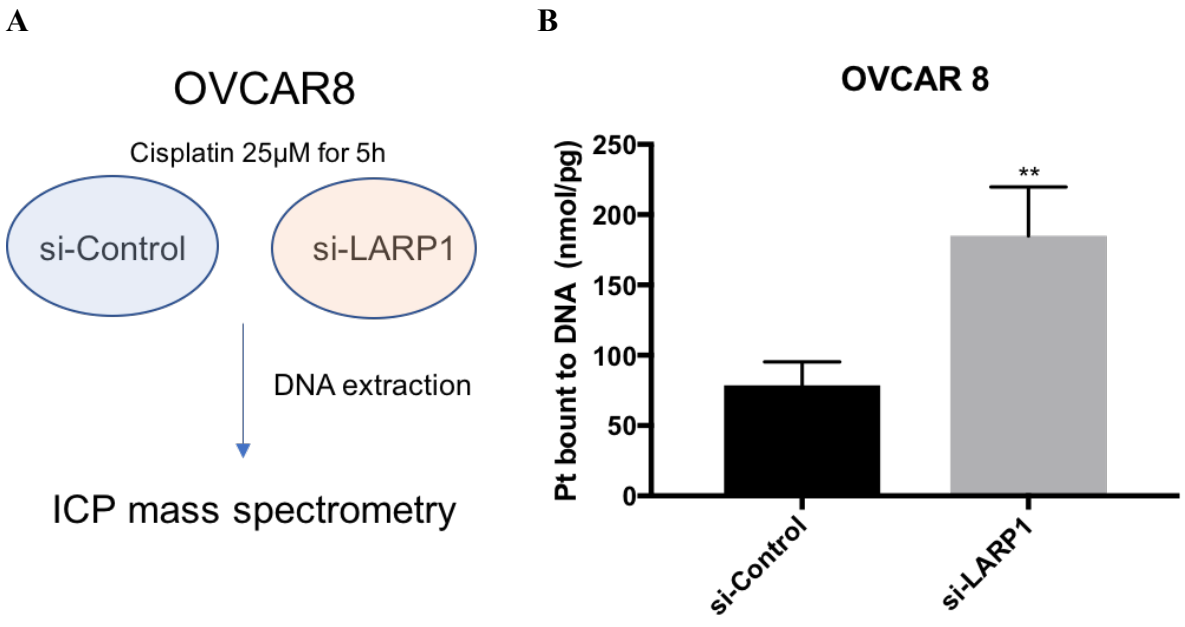


Figure 3-48 LARP1 knockdown increases the formation of cisplatin - DNA adducts.

A. Schematic outlining the experimental process followed.
B. Graph showing the amount of cisplatin bound per picogram of DNA in the cisplatin resistant OVCAR8 cells before and after LARP1 knockdown with siRNA.

3.3.8 SUMMARY

In this sub-chapter I focused on the RNA – dependent interaction between LARP1 and YB-1 and investigated its biological significance by exploring their common mRNA targets. I identified that both proteins are in complex with and regulate the expression of genes involved in cisplatin resistance at a post-transcriptional level. Such genes include the copper efflux transporter ATP7B, the solute carrier family 30 members 1 (SLC30A1) and the damaged DNA binding protein 2 (DDB2). Both proteins were found to upregulate the expression of the copper efflux pump ATP7B while downregulating the expression of DDB2, a protein whose overexpression enhances sensitivity to cisplatin. I focused on the effect of YB-1 and LARP1 on the expression of ATP7B, which is a key mediator of cisplatin resistance, showing that LARP1 knockdown has a greater impact in decreasing the abundance of ATP7B at an mRNA and protein level compared to YB-1 or their concurrent knockdown. This indicates that LARP1, as a component of greater mRNP complexes, is a key post-transcriptional regulator of the expression of genes that confer cisplatin resistance.

Furthermore, I identified that LARP1 and YB-1 are in complex with and regulate the abundance of each other's mRNA. LARP1 upregulates the expression of YB-1 whereas YB-1 downregulates the expression of LARP1. This finding adds an extra level of complexity in the regulatory functions of the YB-1/LARP1 complex on their identified mRNA targets.

3.4 THE EFFECT OF LARP1 KNOCKDOWN ON TUMOUR GROWTH AND ITS SYNERGISTIC ACTION WITH CISPLATIN

3.4.1 IN VITRO ASSAY: TUMOUR SPHEROIDS

In order to assess the effect of LARP1 on tumour growth and investigate its potential synergistic action with cisplatin, I used a 3D culture model of tumour spheroids from SKOV3-derived tetracycline inducible clones (LARP1 shRNA and GFP shRNA). Cells were seeded in ultralow attachment U-bottomed 96-well plates and allowed to grow until tumour spheroids were formed. The clones contained the TET-ON expression system [348] (section 2.13) which allows the expression of the shRNA of interest in the presence of tetracycline. Effective induction of LARP1 knockdown was achieved with tetracycline at a final concentration of 1μg/ml Figure 3-49 (A). The spheroids reached a measurable size on Day 5 post seeding and were divided in four treatment categories as seen in Figure 3-49 (B): 1. No treatment; 2. Tetracycline only; 3. Combination of tetracycline and cisplatin administered simultaneously and 4. Combination of tetracycline and cisplatin, with cisplatin being administered 24h after the tetracycline. The above treatments were administered on Days 5 and 8 post-seeding and measurements were continued until day 14 when all the spheroids appeared to be dissolving.

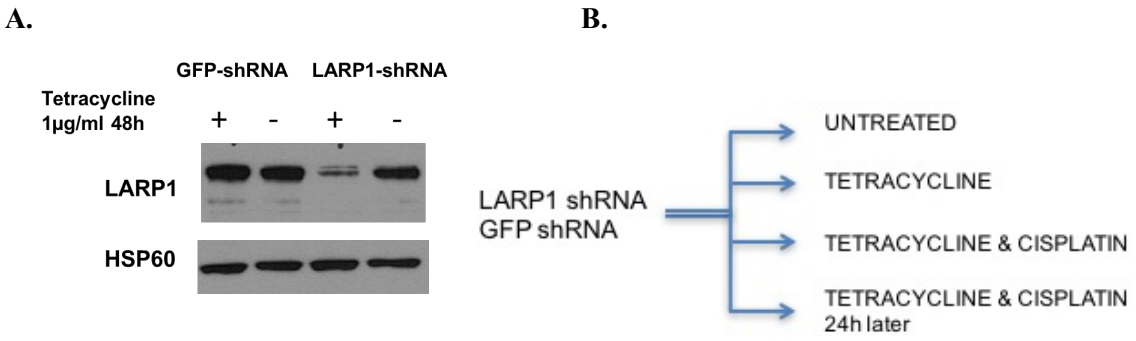


Figure 3-49 In vitro assay with tumour spheroids to assess the synergistic action of LARP1 knockdown with cisplatin

A. Western blot showing effective induction of LARP1 knockdown 48h after treatment with 1µg/ml of tetracycline (Provided by Dr Manuela Mura). **B.** Study schematic

Spheroids were formed from SKOV3 tetracycline inducible (LARP1 shRNA/ GFP1 shRNA/GFP2 shRNA). Control shRNA clones were named GFP1 and GFP2, as the shRNA sequences are to silence expression of green fluorescent protein (GFP), whilst the targeting clone contained an shRNA sequence against LARP1. Five days following seeding they were divided in four treatment conditions: 1. No treatment; 2. Tetracycline; 3. Tetracycline plus CDDP started at the same time and 4. Tetracycline followed by CDDP administered 24h later.

Untreated spheroids kept growing with the LARP1 shRNA clones reaching a relatively smaller final size compared to the GFP shRNA clones. This difference was not statistically significant.

Treatment with tetracycline induced LARP1 knockdown in the LARP1 shRNA clones and resulted in a 15% decrease in their size, compared to the untreated ones. Tetracycline treatment did not have any effect on the size of the GFP shRNA clones showing that LARP1 knockdown exerts a negative effect on tumour growth. Cisplatin treatment led to a decrease in the size of all spheroid cohorts with the greatest effect seen in the LARP1shRNA clones exposed to cisplatin 24h post LARP1 knockdown.

To assess the potential synergistic effect of LARP1 knockdown and cisplatin, I compared the size of tetracycline induced LARP1 shRNA clones and the GFP-shRNA controls upon exposure to cisplatin as shown in Figure 3-50. The combination of cisplatin and LARP1

knockdown was found to be more effective in reducing the tumour spheroid size compared to cisplatin single treatment, reflecting their synergistic action. It is of note that the treatment combination including LARP1 knockdown followed by cisplatin 24h post-knockdown, had the greatest anti-tumour effect compared to their simultaneous administration.

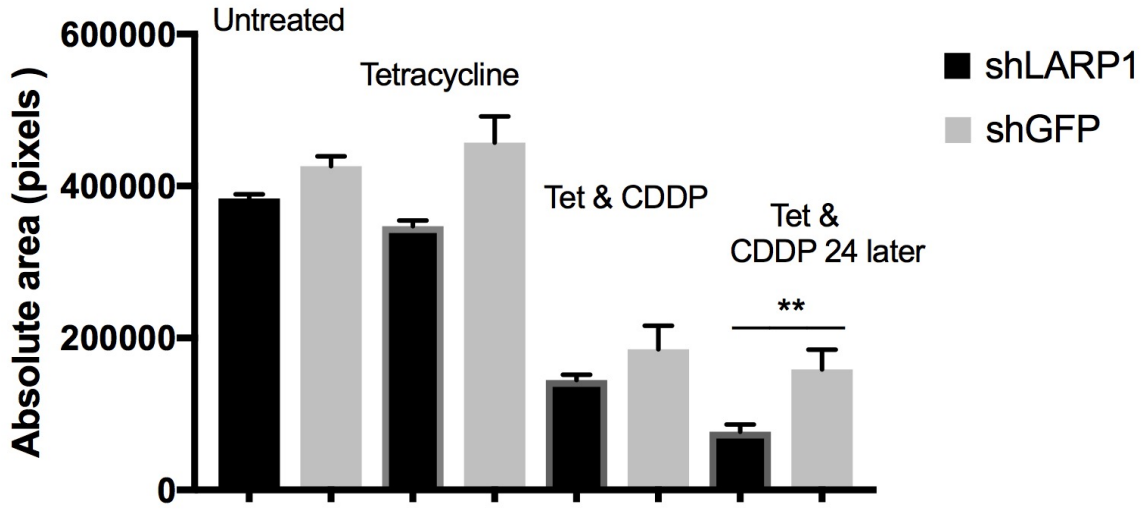


Figure 3-50 Comparison of treatment effect on SKOV3 spheroids' size on Day 10.

Effect of the various treatments on the size of the spheroids on Day 10 post-seeding. LARP1 knockdown and cisplatin demonstrate a synergistic action in reducing the tumour spheroid size compared to cisplatin treatment alone. However, the combination of LARP1 knockdown followed by cisplatin treatment 24h post-knockdown had the greatest effect in reducing the size of the tumour spheroids.
(shLARP1: tetracycline inducible clones; shGFP: controls; tet: tetracycline; CDDP: cisplatin)
(* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

3.4.2 IN VIVO EXPERIMENT

SKOV3 TET-ON inducible clones were injected subcutaneously in a cohort of 4 female BALB/c nude mice as described in the methods section. In order to prevent bias due to host-to-host variation, control (GFP shRNA) and LARP1 (LARP1shRNA) knockdown cells were injected into the right and left flank of each mouse respectively Figure 3-51 **.**

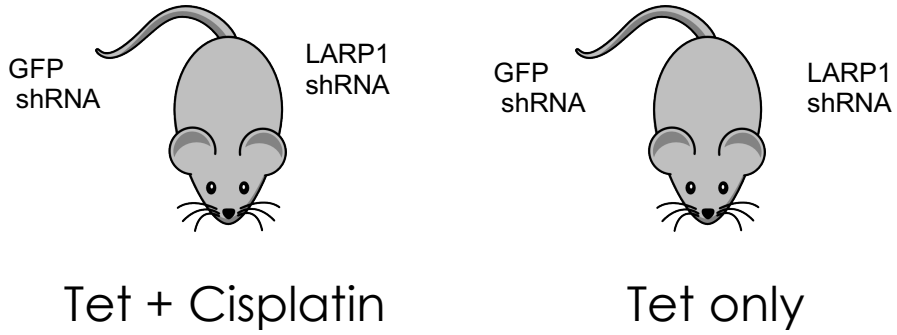


Figure 3-51 *In vivo* study-design schematic.

SKOV3 TET-ON inducible clones were injected subcutaneously in a cohort of 4 female BALB/c nude mice. Control (GFP shRNA) and LARP1 (LARP1shRNA) knockdown cells were injected into the right and left flank, respectively, of each mouse. All mice were treated with doxycycline to induce LARP1 knockdown and 2 also received cisplatin.

Eight weeks after the cancer cell inoculation the tumours reached the pre-defined measurable size of (~50 mm^3) and treatment with doxycycline pellets was commenced in all 4 mice to induce LARP1 knockdown. Cisplatin was administered to 2 of the mice via intraperitoneal injections twice a week as shown in Figure 3-52**.**

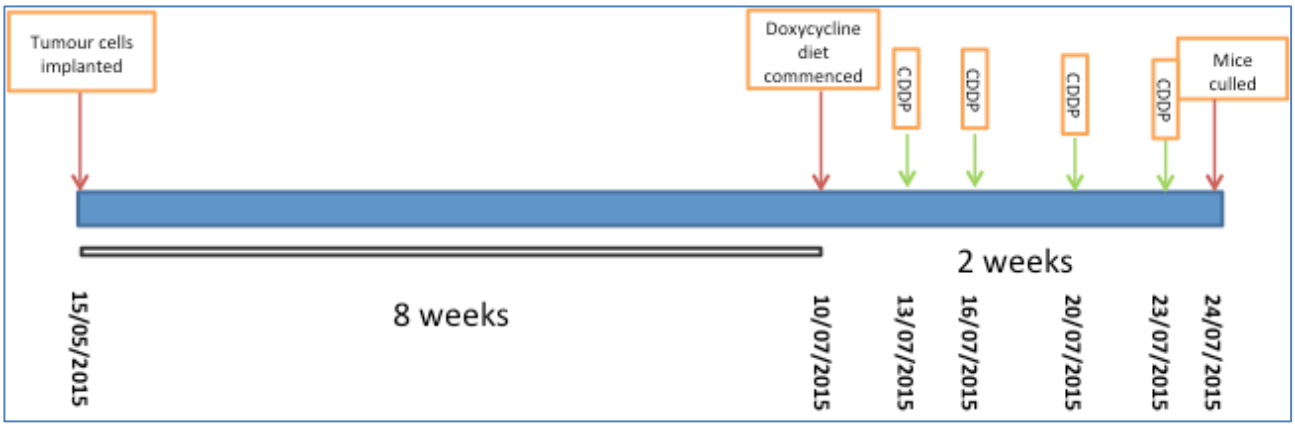


Figure 3-52 *In vivo* study-dosing schematic.

Study schematic showing the timeline for the experiment and the dosing schedule for the administered drugs.

There was a profound delay for the LARP1 shRNA cells to form measurable tumours compared to the control (GFP shRNA) cells even before doxycycline was administered for LARP1 knockdown induction (8 weeks vs 4 weeks). Furthermore, the initial size of the LARP1 shRNA tumours on the day of treatment commencement was smaller compared to (GFP shRNA) controls. In the cohort of the mice treated with doxycycline only, it was evident that LARP1 knockdown led to smaller tumour sizes compared to controls (GFP shRNA) (Figure 3-53 **A)**. The same trend was also observed in the cohort that received doxycycline and cisplatin, showing that a combination of LARP1 knockdown and cisplatin had greater effect at attenuating tumour growth compared to cisplatin alone **(**Figure 3-53 **B)**.

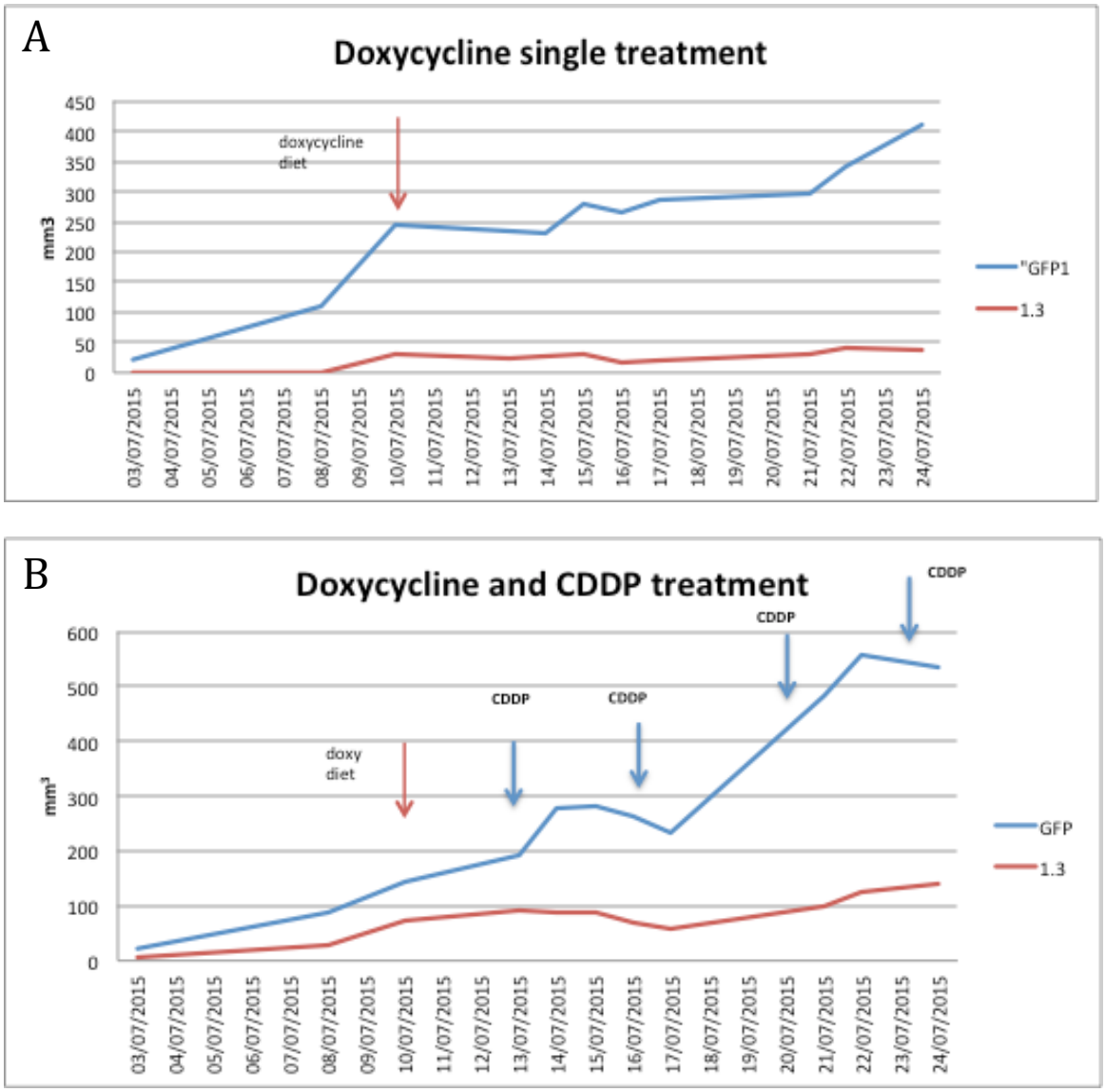


Figure 3-53 *In vivo* study: treatment effect on tumour growth.

Graphs showing the effect of LARP1 knockdown with or without cisplatin treatment on tumour growth. (N= 2 tumours per cohort)

A. The size of tumour xenografts on LARP1 knockdown (red line) compared to control (blue line)
B. The size of tumour xenografts on treatment with a combination of LARP1 knockdown plus cisplatin (red line) versus Cisplatin only (blue line) [1.3 clones: LARP1 shRNA; GFP clones: GFP shRNA; CDDP: cisplatin

4 CHAPTER 4 -DISCUSSION

4.1 DISCUSSION

4.1.1 LARP1 IS INVOLVED IN FUNDAMENTAL CELLULAR PROCESSES

In this project, with the use of co-immunoprecipitation followed by mass spectrometry, I explored the protein interactome of LARP1 in a cisplatin resistant (OVCAR8) and a sensitive (OVCAR3) ovarian cancer line cell, before and after treatment with cisplatin, and further characterised the biological role and functions of such interactions. I found that in both cell lines (in the untreated state) LARP1 is in complex with clusters of proteins comprising the translation machinery (40S and 60S ribosomal subunit proteins, DDX helicases), proteins involved in mRNA processing (heterogeneous nuclear ribonucleic binding proteins) and cell survival. This is consistent with the findings of Burrows et al, who performed LARP1 immunoprecipitation in the ovarian cancer cell lines PE01 and PE04, showing that LARP1 interacts with proteins involved in translation, transcription, cell cycle, as well as with cytoskeletal proteins. Furthermore, in a study by Tcherkezian al [323] aimed to identify the proteins complexed with the mRNA 5' cap in the benign cell line HEK293, LARP1 was found among the immunoprecipitated proteins along with the same functional clusters of proteins as identified in my experiments.

The fact that LARP1 appears to consistently be in complex with the same functional clusters of proteins involved in translation and mRNA metabolism, in both malignant and benign cells, underlines its key role in such cellular functions [327]. The pivotal role LARP1 plays in protein translation as well as in regulating the homeostasis of mRNA transcripts has been described in a number of studies and LARP1 is an established key regulator of 5' TOP mRNA translation [323, 327, 330, 390].

4.1.2 LARP1 CHANGES ITS PROTEIN BINDING PARTNERS UPON CISPLATIN TREATMENT BUT MAINTAINS A CORE COMPLEX IN THE RESISTANT OVCAR8 CELL LINE

In both OVCAR8 and OVCAR3 cell lines, LARP1 changes a number of its binding partners upon cisplatin treatment with a more dramatic change occurring in the sensitive cell line (OVCAR3), where it remains interacting only with few ribosomal and cytoskeletal proteins. In the resistant OVCAR8 cell line, LARP1 acquires more binding partners but also maintains a "stable" complex which is not significantly altered. A similar effect is also noted for the OVCAR3 cell line but to a lesser extent, as the majority of LARP1 protein interactions are lost. It needs to be noted that despite belonging to the same functional category, the individual components of the LARP1 protein complex are not identical in the two conditions (treated vs untreated). One example is that in the untreated OVCAR8 cells LARP1 interacts with several heterogeneous nuclear ribonucleic binding proteins but the hnRNPs K and M only appear to be complexed with LARP1 upon cisplatin treatment.

The proteins YB-1, PAPB1 and PABP4 were identified at the core of the "stable" LARP1 complex observed in the cisplatin resistant OVCAR8 cell line and were further validated as key LARP1 interactors on the basis of their biological functions and high mass spectrometry scores. A key difference noted between the resistant (OVCAR8) and the sensitive (OVCAR3) cell lines was that, upon cisplatin treatment, YB-1 remained in complex with LARP1 only in the OVCAR8 but not in the OVCAR 3 cells. Both LARP1 and YB1 have been found overexpressed in various tumours and cancer cells lines, including ovarian cancer, and have been implicated in promoting cisplatin resistance [332, 369, 396, 397, 401, 417]. This suggests that the LARP1-YB1 interaction could be of great significance in mediating cisplatin resistance in ovarian cancer.

4.1.3 LARP1 LOCALISES IN THE NUCLEUS FOLLOWING CISPLATIN TREATMENT

At a steady state LARP1 is predominantly cytoplasmic, but becomes nuclear following treatment with cisplatin. This feature was consistently observed in all the investigated ovarian cancer cell lines regardless of their sensitivity or resistance to cisplatin. Having tested a number of chemotherapeutic agents, I identified that this effect was only triggered upon treatment with cisplatin or bleomycin, which both induce DNA damage, but not by paclitaxel or gemcitabine which do not target the DNA. This indicates that LARP1 plays a key role in stress response but its functions vary depending on the type of cellular stress, as for example, treatment with sodium arenite results in its accumulation in stress granules but not in the nucleus [332]. The nuclear localisation of LARP1 upon cisplatin may partly explain the changes that occur to its binding partners as identified by mass spectrometry. In the treated cells LARP1 interacts with proteins involved in stress response and splicing and loses some of its ribosomal protein partners. The fact that LARP1 maintains interaction with ribosomal proteins following cisplatin treatment suggests that it likely forms several complexes and gets involved in different cellular processes in order to maintain the cellular homeostasis.

Cytoplasmic LARP1 is known to be involved not only in mRNA translation, but also in regulating the stability of transcripts encoding for survival and apoptotic factors [323, 325, 332]. A nuclear role for LARP1 in response to genotoxic stress remains unknown but could include splicing [418], or the nucleocytoplasmic transportation of new mRNAs to be translated. DNA damage is found to affect the cellular localisation and activity of splicing factors via post-translational modifications [419]. Since LARP1 interacts with known splicing factors such as YB-1 as well as hnRNPs (such as hnRNP A1), it is possible that it may be translocated in the nucleus to be involved in splicing [395, 420]. This supports the

assumption that LARP1 is a versatile multifunctional protein which changes its subcellular localisation and binding partners in response to genotoxic stress.

The mechanisms underpinning the subcellular translocation of LARP1 still remain vague. LARP3 (Genuine protein La) is known to translocate from the nucleus to the cytoplasm following Akt phosphorylation [293] or cleavage in response to apoptotic stimuli induced by chemotherapy or UV-radiation [294]. Therefore, one could speculate that a similar mechanism could also mediate the subcellular translocation of LARP1. Both genuine La and LARP6 [421] carry nuclear localisation signals but the presence of such region in LARP1 has not yet been mapped.

It is not yet known whether the nucleocytoplasmic shuttling of LARP1 is dependent on its interactions with other proteins. Proteins of the 14-3-3 family were identified in complex with LARP1 by mass spectrometry and they could facilitate its nuclear transport [422] . It is also possible that it could be mediated by its interaction with PABP1 and/or YB-1, which have also been found to shuttle from the cytoplasm to the nucleus in response to DNA damaging factors [359, 375, 395, 399]. YB-1 has been reported to translocate to the nucleus following cellular exposure to cytotoxic stimuli including UV irradiation and cisplatin [367, 399] and its nuclear localisation has been associated with adverse survival outcomes and drug resistance in several types of cancer including ovarian [368-370]. In the nucleus, it is believed to act either as transcription factor or to modulate other transcription factors in order to promote the expression of genes required to overcome the stress [395]. Such an example is the transcriptional activation of the multidrug resistance (MDR1) as well as the major vault protein (MVP/LRP) genes which are involved in acquisition of global drug resistance [365, 423, 424]. Apart from acting as a transcription factor, YB-1 also interacts with damaged DNA promoting its repair and protecting it from the cytotoxic effects [425]. Since LARP1 has not yet been found to have DNA binding capacity, it comes with hesitation to speculate

that it could act as a transcriptional activator or mediate DNA repair, but this would be of interest to explore. The nucleocytoplasmic shuttling of YB-1 has also been associated with the formation and transport of mRNPs to the cytoplasm and a similar function has also been recognised for PABP1 [375, 426]. LARP1 colocalises with YB1 in the cytoplasm of untreated OVCAR8 and OVCAR3 cells. Upon cisplatin, the colocalisation becomes more nuclear in the OVCAR8 cell line, whereas in the OVCAR3 line the two proteins colocalise predominantly at in the nuclear rim. Despite their colocalisation, upon cisplatin the interaction between LARP1 and YB-1 is only maintained in the resistant OVCAR8 cell line. Having demonstrated that this interaction is RNA dependent, it is likely that LARP1 and YB-1 share common mRNA targets which they preserve during genotoxic stress in the OVCAR8 but not in the OVCAR3 cell line. This could be because they change their mRNA targets or because their common mRNA targets are not transcribed in the OVCAR3 cells following cisplatin. It is possible that these two key mRNA binding proteins are components of mRNPs containing transcripts that are necessary for maintaining cell survival upon cisplatin treatment.

The colocalisaton of LARP1 and PABP1 was also investigated with immunofluorescence and both proteins were found to colocalise in the cytoplasm of the untreated OVCAR8 and OVCAR3 cells. Following cisplatin treatment, LARP1 colocalises with PABP1 in the nucleus of the cisplatin sensitive (OVCAR3) cells only. In the resistant (OVCAR8) cell line PABP1 remains in the cytoplasm where it still maintains a degree of colocalisation with LARP1, whereas the majority of LARP1 is found in the nucleus. To further explore the pattern of localisation of PABP1 upon cisplatin, I performed immunofluorescence in the isogenic A2780 (sensitive) and A2780-CP70 (resistant) cell lines. Again, PABP1 remained predominantly cytoplasmic post cisplatin treatment in the resistant cells (CP70) but became nuclear in the sensitive ones (A2780). (data not shown). This observation could reflect the

heterogeneity among different cell lines. However, this could also be a pattern suggesting that in the resistant lines PABP1 remains in the cytoplasm, following genotoxic stress, as translation is maintained (as confirmed by the SuNSET experiment). On the other hand, translation is disrupted by cisplatin in the sensitive cell lines and therefore PABP1 may obtain other functions. As there is a remaining amount of cytoplasmic LARP1 colocalising with PABP1 in the treated resistant cells lines, this could suggest that it may as well be involved in the translation of transcripts required for the cell to survive the stress.

It has been demonstrated that poly (A) mRNA distribution governs the subcellular localisation of cytoplasmic PABP1 [384]. A reduction in protein synthesis as well as perturbations that block mRNA export following UV exposure, were found to retain PABP1 within the nucleus. Transcriptional inhibition also had the same effect on PABP1[359, 375]. It is possible that the subcellular localisation of LARP1 is directed by a similar RNA dependent mechanism and this needs further investigation.

All things considered, I speculate that LARP1 holds multiple roles within the cell and can dynamically change its functions (and binding partners) to maintain cellular homeostasis through genotoxic stress. At a resting state LARP1, along with PABP1 and YB-1 are cytoplasmic, and could be predominantly involved in the translation or post-transcriptional regulation of mRNA transcripts. Upon genotoxic stress, LARP1 becomes predominantly nuclear and is likely to obtain new functions and facilitate the cell response to genotoxic stress. YB-1 accompanies LARP1 in the nucleus of cisplatin resistant cells and they share common mRNA targets. Their RNA-dependent interaction is only preserved in cisplatin resistant cells and could play an important role in the post-transcriptional regulation and the nucleocytoplasmic transport of transcripts vital for cell survival.

4.1.4 LARP1 FORMS AN RNA-MEDIATED COMPLEX WITH YB-1 AND PABP1

I identified that LARP1 interacts with the two major RNA binding proteins YB-1 and PABP1 and they all co-immunoprecipitated with each other suggesting that they are part of the same complex. Despite the fact that the interactions between LARP1 and PABP1 and well as PABP1 and YB-1 have been previously reported, this is the first report of the three proteins co-immunoprecipitating in an ovarian cancer cell line. I proved that all the interactions among the three proteins are RNA-dependent demonstrating their disruption following treatment with Micrococcal nuclease. This nuclease digests both RNA and DNA leaving mono and di-nucleotides, and contrary to RNAse A, does not entail the bias of leaving undigested poly A tails.

An interaction between LARP1 and PABP1 has been previously described in a number of studies involving benign (HEK293) and cervical cancer (HeLa) cell lines with conflicting outcomes regarding its direct or indirect nature [323-325, 330]. Aoki et al, suggested an indirect RNA-dependent interaction between LARP1 and PABP1 which was disrupted with RNAse I, a nuclease that cleaves after each nucleotide base [324]. In contrast, Burrows et al [330] favoured a direct interaction of these two proteins since it was maintained upon digestion with RNAse. Despite the fact that the digestion was done at 4 ^{o}C, RNAse A cleaves preferentially after pyrimidine nucleotides which could leave the poly A tails intact. Tcherkezian et al [323] supported a direct interaction which was also considered to mediate the recruitment of LARP1 in the mRNA 5'cap and polysomes. To identify the interacting region of the two proteins, C-terminal deletion mutants were constructed lacking a stretch of DM15 tandem repeats (LARP1 Δ150) or all DM15 tandem repeats (LARP1Δ300). In both cases the interaction between LARP1 and PABP1 was lost suggesting that the DM15 region mediates the interaction. However, a more recent study by Berman et al [260], demonstrated that the C-terminal DM15 region is an RNA binding motif which binds directly 5' TOP

sequences. Deleting stretches within this RNA binding region could inevitably affect the RNA binding capacity of LARP1 and consequently a potential RNA-dependent interaction with PABP1. On a similar note, Fonseca et al [325] suggested that LARP1 interacts with PABP1 directly via a 11-aminoacid PAM2-like motif located within the La motif as its deletion compromised the interaction. However, the La motif is well known to have RNA binding properties [257] and therefore interfering with this region could possibly compromise the RNA binding capacity of LARP1 and therefore affect a potential RNA binding interaction. It is possible that PABP1 and LARP1 interact directly and this interaction is further enhanced by RNA. This has been previously demonstrated for PABP1 and eIF4G [427]. Safaee et al, used crystallography studies to demonstrate the interaction of eIF4G with the RRM2 domain of PABP1. They also showed that this interaction was allosterically regulated by the presence of poly(A) RNA bound to PABP1 [427]. A similar mechanism could underpin the interaction between LARP1 and PABP1.

Interestingly, while YB-1 has been an extensively studied multifunctional protein involved in carcinogenesis, very little is known about its interaction with LARP1 particularly in human cell lines. LARP1 has been previously identified as a component of the YB-1 ribonucleoprotein complex involved in the replication of the hepatitis C virus [428]. It is of note that in YB-1 pull downs performed in the colorectal (SW480) and the breast (MCF7) cell lines in the presence of RNAse A, PABP1 was identified as a direct YB1 interactor but not LARP1. Despite the fact that the experiment was performed in different cell lines compared to mine, this could re-inforce my finding that the LARP1 - YB-1 interaction is RNA mediated. However, a direct interaction between PABP1 and YB-1 contradicts my findings and may be attributed to the differences among the investigated cancer cell lines or the experimental procedures followed.

All three proteins are master mRNA regulators and play a pivotal role in the post-transcriptional regulation and translation of mRNA transcripts [394]. Their RNA-dependent interactions indicate that they share and consequently regulate some common mRNA targets. The role of PABP1 in stability and translation of all the mRNAs bearing polyadenylate tails has been extensively investigated [358]. Both LARP1 and YB-1 are known to selectively regulate the stability and decay of mRNA transcripts associated with cell proliferation, tumorigenesis and cisplatin resistance [332, 395]. The preservation of the interaction between LARP-1 and YB-1, upon cisplatin treatment, only in the cisplatin resistant OVCAR8 cell line supports the hypothesis that they could play a key role in regulating the expression of mRNA transcripts required for cell survival following cisplatin-induced genotoxic stress. In fact, both proteins have been found to promote the expression of BCL2 and enhance cell survival in malignant cell lines [402].

Using the Duolink proximity ligation assay, LARP1 was found to be in close proximity with YB-1 and PABP1, which could mean that their binding sites on their mRNA targets are also in proximity. LARP1 is known to interact with the 3' untranslated regions (3' UTRs) of BCL2, BIK and mTOR mRNA transcripts, stabilising the first and destabilising the latter [332]. Furthermore, LARP1 has been found to interact with the 3' and 5' UTRs of 5 TOP mRNAs regulating their stability and translation [323, 390].

Identifying the common target transcripts of LARP1 and YB-1 as well as their RNA binding motifs would be of paramount importance in order to better understand the mechanism of their involvement in cisplatin resistance.

4.1.5 THE LARP1 - YB1 COMPLEX REGULATES THE EXPRESSION OF GENES INVOLVED IN PRE- AND POST-TARGET MECHANISMS OF CISPLATIN RESISTANCE

I explored the common mRNA targets of LARP1 and YB-1 by cross-referencing published databases and identified that they both regulate the mRNA abundance of a subset of genes involved in cisplatin resistance including the copper efflux pump *ATP7B*, the damaged DNA binding protein subunit 2 (*DDB2*), the soluble carrier factor *SLC30A1* and the anti-apoptotic factor BCL2 which is a known LARP1 target [332, 403]. With the use of RNA immunoprecipitation and RT-PCR, I validated that both LARP1 and YB-1 are complexed with these mRNA transcripts [332] . The overexpression of *ATP7B* has been associated with cisplatin resistance in number of malignancies, including ovarian cancer, where it has also been identified as a potential therapeutic target [127, 129, 429]. The role of the zinc transporter protein *SLC30A1* in cisplatin resistance has not yet been fully defined, however its expression has been found increased in response to cisplatin in ovarian and bladder cancer cells [405]. Furthermore, overexpression of the damaged DNA binding protein subunit 2 (*DDB2*) has been associated with sensitisation of ovarian cancer cell lines to cisplatin via the activation of caspase pathway and downregulation of BCL2 protein [406]. Both LARP1 and YB-1 knockdown resulted to a decrease in the mRNA abundance of *ATP7B* and increased the abundance of *DDB2*. However, they exerted different effects on *SLC30*A1 mRNA, which was downregulated by LARP1 knockdown, while being upregulated by YB-1 knockdown. *ATP7B* was further investigated in this project due to its recognised importance in mediating cisplatin resistance.

YB-1 has been previously reported to stabilise mRNA transcripts when binding in conjunction with other RNA binding proteins such as nucleolin [430]. LARP1 is known to have a differential effect on its target mRNAs, which could also be jointly mediated by other RNA binding proteins [332]. It is likely that LARP1 and YB-1, act as key components of

mRNP complexes containing mRNAs involved in cisplatin resistance and regulate their expression by stabilising or destabilising them. It is yet to be defined whether the effect on their target transcripts is exerted mainly by their own binding or whether they act as scaffolding proteins and recruit more RBPs. Such an example is that binding of both YB-1 and nucleolin is required for the stabilisation of interleukin 2 mRNA [430]. Interestingly, nucleolin has been found enriched in LARP1 pulldowns in HeLa cells, but was not present in the immunoprecipitations performed in ovarian cancer cell lines. However, this indicates that LARP1 and YB-1 may also act in complex with other RBPs to exert their role and this warrants further investigation.

All the above evidence supports a potential role of the LARP1-YB1 complex in regulating the stability of their target transcripts. However, it is possible that they may also be involved in other levels of their post-transcriptional regulation. I have demonstrated that both LARP1 and YB-1 become nuclear in response to genotoxic stress in cisplatin resistant cells and this could indicate their involvement in other steps of post-transcriptional regulation. YB-1 is known to be involved in splicing as well as in mRNA packing for nuclear export [395]. Such functions are yet to be confirmed for LARP1. The exact post-transcriptional mechanism (splicing, nucleocytoplasmic transfer, stability or translation) by which these two proteins regulate their target mRNAs is uncertain, however the net effect of this regulation is promotion of cisplatin resistance.

Here, I show that the LARP1 – YB-1 complex promotes cisplatin resistance by post-transcriptionally regulating genes involved in pre-and post-target resistance mechanisms. Via upregulating the expression of the efflux transporter *ATP7B*, it can decrease the net amount of cisplatin uptake in the tumour cells (pre-target resistance). This was further validated for LARP1 as I demonstrated that its knockdown with RNA interference increased the amount of DNA-bound cisplatin. LARP1 and YB-1 were also found to downregulate the expression of

DDB2 which promotes apoptotic signals while upregulating the anti-apoptotic factor *BCL2* (post-target resistance). The upregulation of *BCL2* expression, which plays a key role in post-target resistance, has also been previously proven for both LARP1 and YB-1 [332, 402]. The fact that the LARP1- YB-1 complex can possibly mediate cisplatin resistance via its involvement in pre-and post–target mechanisms highlights its significance as a potential therapeutic target.

4.1.6 LARP1 AND YB1 RECIPROCALLY REGULATE THEIR OWN TRANSCRIPTS

LARP1 has been previously found in complex with YB-1 mRNA, upregulating its levels [327]. Here, I discovered that YB-1 is also in complex with LARP1 mRNA and downregulates its expression. It is of note that double knockdown of LARP1 and YB-1 resulted in a less effective decrease in their respective mRNA levels compared to that achieved with each one's individual knockdown. In the case of LARP1, this could be attributed to the fact that YB-1 knockdown leads to an increase in LARP1 levels and therefore the net effect of the double knockdown is reflected in the observed LARP1 levels. However, in the case of YB-1, a greater decrease in its abundance would have been expected upon double knockdown. This could potentially indicate that the simultaneous depletion of these two key mRNA regulators triggers the activation of other compensatory mechanisms in cancer cells in order to maintain their expression.

The reciprocal regulation of LARP1 and YB-1 on their own transcripts and the opposite effects they exert on each other's abundance adds further complexity in the regulation of their common mRNA targets. In regards to the expression of ATP7B, LARP1 knockdown was more effective in decreasing its mRNA levels compared to single YB-1 or double knockdown of both proteins. This could be due to the fact that LARP1 knockdown also results in a decrease of YB-1 abundance and therefore has a combined effect on the expression of

ATP7B. The regulatory effect of LARP1 and YB-1 on each other's expression needs to be further investigated and taken into account when the therapeutic potential of targeting the LARP1 - YB-1 complex is considered.

4.1.7 THE LARP1- YB1 COMPLEX AS A POTENTIAL THERAPEUTIC TARGET

In this project, I have identified that LARP1 and YB-1 are key components of mRNPs containing transcripts involved in pre-and post-target mechanisms of cisplatin resistance. Such transcripts include the ATP7B efflux pump as well as the damaged DNA binding protein subunit 2 (DDB2) and the anti -apoptotic factor BCL2. Via their concurrent binding on these transcripts they differentially affect their expression at a post-transcriptional level. Preservation of the LARP1-YB1 interaction with their common mRNA targets was found to be of particular significance for resistant ovarian cancer cells (OVCAR8) to overcome cisplatin induced genotoxic stress and should be investigated as a potential therapeutic target. Identifying the RNA interactome of the LARP1-YB1 complex as well as the RNA binding motifs of YB-1 and LARP1 would be of paramount importance in order to reveal a potential regulatory mechanism. Each protein's binding with identified mRNA targets of interest could then be inhibited with the use of aptamers in order to elucidate a potential mechanism of action. Another possible therapeutic approach would be to target the expression of LARP1, YB-1 or both with the use of RNA interference.

4.1.8 LARP1 KNOCKDOWN HAS A SYNERGISTIC ROLE WITH CISPLATIN IN OVARIAN CANCER CELL LINES

LARP1 knockdown has already been found to have a synergistic effect with cisplatin, resulting in a significant increase in the apoptosis of resistant cells [332]. In this project, I identified that a combinational treatment of the resistant OVCAR8 cells with cisplatin and LARP1 knockdown, resulted in a greater degree of apoptosis compared to that noted with

YB-1 knockdown or double LARP-1/YB-1 knockdown (Appendix 6.2). This could be partly attributed to the fact that LARP1 knockdown had a greater effect than YB-1 or their double knockdown, in decreasing the expression of the copper efflux pump ATP7B, which consequently results in increased genotoxic damage. Whether a similar effect is also noted for other mRNA targets such as the anti-apoptotic factor BCL2 needs to be further investigated.

I further validated the synergistic effect of LARP1 with cisplatin in 3D *in-vitro* and *in vivo* models using a tetracycline-inducible system in cisplatin resistant ovarian cancer SKOV3 cells. The synergistic effect of LARP1 knockdown and platinum was assessed with 3D *in vitro* as well as with *in vivo* models. In the *in vitro* model a sequential combination of LARP1 knockdown and cisplatin led to a significant decrease in tumour size compared to cisplatin single agent. In the *in vivo* experiment, there was a trend showing that a combination of LARP1 knockdown and cisplatin had greater effect at attenuating tumour growth compared to cisplatin alone. Apart from its limited sample size, the *in vivo* experiment was also limited by the fact that there was a significant delay in the LARP1 shRNA clones to reach a measurable size compared to the control (GFP-shRNA) clones. This was most likely attributed to a leak of LARP1 shRNA in the TET-on system even before the doxycycline administration [431]. In order to tackle this issue this in vivo experiment is currently repeated by our collaborators (Professor Anil Sood and team) at the MD Anderson using biocompatible DOPC nanoparticles containing siRNA to LARP1. Preliminary data have so far shown that LARP1 knockdown restores sensitivity to cisplatin resistant ovarian cancer xenografts.

It has to be taken into account that the use of RNA interference could affect cell viability and apoptosis due to off-target effects [432]. In this project, I used two siRNAs and an shRNA construct against LARP1 obtaining similar outcomes.

4.2 FUTURE WORK

4.2.1 IDENTIFY THE RNA MOTIF AND GLOBAL RNA INTERACTOME OF LARP1

So far, the mRNAs complexed with LARP1 have been identified in the HeLa cell line with the use of RNA immunoprecipitation and microarray profiling (RIP-Chip) and few targets have been validated in the OVCAR8 cell line. In a recent study the mRNA interactome of LARP1 was explored with the use of photoactivatable ribonucleoside–enhanced crosslinking and immunoprecipitation (PAR-CLIP) in the benign HEK293 cell line, demonstrating the enrichment of LARP1 with mRNAs encoding for factors involved in translation [390]. A similar study has been done for YB-1, a key protein interactor of LARP1, in which a new function of YB-1 was discovered in the regulation of non-coding RNAs and particularly miRNAs with implications in tumorigenesis [433]. In this project, I identified that in the cisplatin resistant OVCAR 8 cell line the majority or LARP1 interactions with other proteins are RNA-dependent, which underlines the importance of exploring its RNA interactome. YB-1 was identified as a key interactor of LARP1 and both proteins are key components of mRNPs containing mRNAs of genes involved in mechanisms of cisplatin resistance. It is of paramount importance to investigate not only the transcriptomic changes occurring in cisplatin resistant and sensitive ovarian cancer cells before and after cisplatin, but also the mRNA targets of LARP1 and YB-1 in each condition. This would potentially result in the identification of all important mRNA targets that mediate cisplatin resistance. UV-mediated crosslinking and immunoprecipitation coupled with RNA sequencing, such as i-CLIP (individual nucleotide resolution cross linking and immunoprecipitation), for both LARP1 and YB-1 can reveal direct RNA-protein interactions in the whole transcriptome [434] and identify RNA binding motifs for LARP1 and YB-1.

LARP1 has already been found to act on the 3'UTRs of transcripts such as BIK, BCL2 and mTOR and differentially affecting their abundance, stabilising BCL2 and mTOR and destabilising BIK transcript to promote tumorigenesis [327, 332]. This differential effect on its targets is likely to be mediated by the concurrent binding of other RBPs. In this project, I identified that LARP1 and YB-1 regulate the abundance of their common transcripts exerting either a similar or an opposite effect, which is likely to be determined by their binding in a temporal and spatial manner. A detailed understanding of the binding sites of LARP1 and YB-1 on their key mRNA targets and the effect these interactions have on the expression of such targets, would allow the development of novel therapeutic approaches to resensitise ovarian cancer cells to cisplatin.

4.2.2 DETERMINE THE ROLE OF LARP1-YB1 COMPLEX IN THE POST-TRANSCRIPTIONAL REGULATION OF ITS TARGETS

In this project, I have so far identified that LAR1 and YB-1 are in complex with transcripts of genes involved in cisplatin resistance (as key components of their mRNPs) and regulate their abundance. I particularly investigated their effect on the expression of copper efflux pump *ATP7B*, which was upregulated by both proteins. However, it is yet to define the exact role of LARP1 and YB-1 in the post-transcriptional regulation of *ATP7B*.

The role of LARP1 and YB-1 in regulating the stability of their mRNA targets has been already described [327, 332, 430]. Given this, a similar regulatory mechanism for the regulation of ATP7B is highly likely. This speculation could be investigated with a stability assay using actinomycin D to halt de novo RNA transcription and measure the abundance of the *ATP7B* mRNA before and after LARP1 and YB-1 knockdowns. A decrease in the abundance of the *ATP7B* mRNA post LARP1 or YB-1 knockdown, would indicate whether each protein's presence is critical and would also indicate the impact each one has in regulating the expression of the transcript.

Although regulation of their targets' stability is a potential post-transcriptional mechanism for LARP1 and YB-1 to control the expression, one should take into account that these proteins translocate to the nucleus upon genotoxic stress. As RNA stability and decay occur in the cytoplasm, it is likely that the LARP1-YB1 complex also undertakes different functions in the nucleus. They could possibly be involved in splicing or even act as chaperone proteins shuttling mature mRNAs to the cytoplasm to be translated and these functions should be further explored.

In this project, I have established the regulatory effect of LARP1 and YB1 on their identified targets in the cisplatin resistant OVCAR8 cell line. It is crucial to validate these findings in other resistant ovarian cancer cell lines.

4.2.3 EXPLORE THE MECHANISM OF LARP1 SUBCELLULAR TRANSLOCATION UPON CISPLATIN TREATMENT

To better understand the functional implications of LARP1 relocalisation in response to cisplatin induced DNA damage, it would be necessary to identify the underlying mechanism that drives it. Live trafficking of fluorescent tagged LARP1 with confocal microscopy during cisplatin treatment would be very useful to study its localisation at different time points. PABP1 and YB-1, the identified binding partners of LARP1, are also known to shuttle in and out of the nucleus following UV-mediated genotoxic stress and cisplatin treatment respectively [365, 367].

Cellular distribution of poly (A) mRNA was identified as the determinant of PABP1 localisation following perturbations in transcription and consequently translation caused by UV-irradiation. A similar mechanism may also apply for LARP1. This could be investigated by immunofluorescence and confocal microscopy with the use of tagged mRNA and co-localisation studies with LARP1 upon genotoxic stress.

YB-1 nuclear shuttling is known to be mediated by Akt phosphorylation [403]. It is not yet known whether LARP1 undergoes any post-translational modification but this could be of importance to explore. This can be achieved by silico identification of possible phosphorylation sites followed by a confirmatory mass spectrometry [435]. Mutational analysis can then identify whether the identified sites are functionally important.

Another possibility is that the nuclear translocation of LARP1 may be passive due to its interaction with other proteins. From the mass spectrometry proteomic analysis, LARP1 was also found to interact with proteins of the 14-3-3 family which are involved in nucleocytoplasmic transport [422]. This potential interaction could be further investigated and validated.

Once the mechanism underpinning the LARP1 nucleocytoplasmic shuttling is established, it should be further investigated for a potential role in mediating cisplatin resistance. It could be that LARP1 exerts nuclear functions that are crucial for cell survival under genotoxic stress, therefore disrupting its nucleocytoplasmic shuttling could be another potential therapeutic target.

4.2.4 EXLORE THE POTENTIAL DNA BINDING CAPACITY OF LARP1

I have demonstrated that LARP1 relocates from the cytoplasm to the nucleus in response to genotoxic stress and this was observed in both cisplatin resistant and sensitive cell lines. This phenomenon was only observed following treatment with chemotherapeutic agents that target the DNA, therefore it is specific to genotoxic stress. LARP1 was found to be in complex with YB-1, presumably as components of the same mRNPs, and they both follow a similar pattern of subcellular translocation in response to cisplatin. YB-1 is known to have both a DNA and RNA binding capacity and is involved in the transcriptional activation of genes promoting dug resistance such as the MDRI as well as in the repair of DNA damage binding to cisplatin-

damaged DNA [365, 436]. It would be interesting to explore whether LARP1 has a similar role. Chromatin immunoprecipitation followed by high through-put sequencing (CHIP-Seq) [437] would allow the identification of genome-wide LARP1-DNA interactions which could be further validated with electrophoretic mobility shift assays (EMSAs). In CHIP-Seq the protein-DNA complexes are crosslinked with the use of formaldehyde prior to getting sonicated into fragments and immunoprecipitated with anti-LARP1 antibody. The cross-links are then reversed and the DNA segments analysed with deep sequencing.

4.2.5 INVESTIGATE THE DIRECT PROTEIN INTERACTIONS OF LARP1

In this project, I performed RNA digestion with the use of MNase in order to identity the RNA independent protein interactors of LARP1 in the presence and absence of cisplatin in the resistant OVCAR8 cell line. I discovered that only 15% of its protein interactions are RNA independent and these mainly involve ribosomal proteins in the untreated and cytoskeletal proteins in the cisplatin treated cells. Identifying and validating key LARP1 direct protein interactors is rather important to further understand the cellular functions of LARP1 and particularly its role in genotoxic stress response.

4.2.6 DISSECT THE ROLE OF LARP1 IN PROTEIN TRANSLATION

The functional proteomic analysis performed in this project suggests a key role for LARP1 in protein translation via its interaction with ribosomal proteins, as well as with proteins like PABP1 that hold a central role in the process. This role has been further supported in previous studies in which LARP1 was identified as an important regulator of transcripts that encode for ribosomal proteins such as 5'TOP mRNAs [323, 390]. This indicates that LARP1 affects translation in several ways. Protein synthesis is a vital process for cell growth and proliferation and its impaired regulation is a crucial component for cancer development as

well as cell survival during genotoxic stress through the overexpression of certain transcripts [188]. This is reflected in the SUnSET assay done by Dr Mura which shows that upon cisplatin treatment, de novo translation is disrupted in the sensitive OVCAR3 cell line but sustained in the resistant OVCAR8. However, a combination of cisplatin along with LARP1 knockdown, results in a dramatic decrease in translation of the resistant cell line indicating the significant role of LARP1 in the process. It would therefore be of great interest to dissect the role of LARP1 in translation in the context of mediating cell survival during genotoxic stress. A first step would be to identify the transcripts whose translation is promoted by LARP1 in order to maintain cell survival during genotoxic stress. These could be explored by ribosome profiling (or Ribo-Seq) [438] in the presence and absence of LARP1 upon cisplatin treatment. Ribosome profiling allows the identification of the mRNAs that are actively translated at a given time (translatome). Cross referencing these data with the data acquired from the iCLIP experiment will provide a better insight on the role of LARP1 in promoting cisplatin resistance.

4.3 CONCLUSIONS

There is increasing evidence linking RNA binding proteins to tumorigenesis and chemotherapy resistance via the post-transcriptional regulation of gene expression[192, 197]. LARP1 is an RNA binding protein highly expressed in ovarian cancer and acting as an oncogene promoting tumorigenesis and cisplatin resistance [327, 332]. LARP1 knockdown has been found to resensitise resistant ovarian cancer cells to cisplatin, which makes it a promising novel therapeutic target [332]. I further validated this finding in vitro and in vivo using a tetracycline induced 3D culture model as well tumour xenografts.

In this project, I identified that LARP1 is a multifunctional protein which forms several protein complexes and is involved in fundamental cellular processes such as protein translation and mRNA processing. Using the OVCAR8 and OVCAR3 cell lines as models of cisplatin resistance and sensitivity respectively, I discovered that LARP1 forms an RNA mediated complex with PABP1 and YB-1. By immunofluorescence microscopy, I identified that in resting cells (sensitive or resistant) all three proteins are cytoplasmic. Upon genotoxic stress LARP1 and YB-1 become predominantly nuclear in the cisplatin resistant cells whereas PABP1 remains cytoplasmic. A different pattern is noted for the sensitive cells in which PABP1 along with LARP1 become nuclear, whereas YB-1 is localised predominantly in the perinuclear rim and the cytoplasm.

In agreement with its subcellular relocalisation, I discovered that upon cisplatin treatment LARP1 changes a number of its protein interactors with a more dramatic change occurring in the sensitive cell line. I identified that the interaction among LARP1, YB-1 and PABP1 is preserved upon cisplatin treatment in the resistant cell line, but not in the sensitive one where the interaction between LARP1 and YB-1 is lost. Based on this finding and the established involvement of YB-1 in promoting cisplatin resistance, the interaction between LARP1 and YB-1 was further investigated. Both proteins were identified as key components of mRNPs

containing transcripts of genes involved in pre- and post-target mechanisms of cisplatin resistance such as the copper efflux ATPase (ATP7B), the DNA damage binding protein 2 (DDB2) and BCL2. LARP1 and YB-1 act as post-transcriptional regulators and differentially modulate the expression of these targets, upregulating ATP7B and downregulating DDB2. Furthermore, it has been previously shown that LARP1 and YB-1 both upregulate the expression of BCL2 [332, 402]. This results in a decrease in the intracellular concentration of cisplatin (pre-target resistance) as well as an increase in anti-apoptotic signals (post-target resistance) and ultimately cell resistance to cisplatin. Interestingly, I discovered that LARP1 and YB-1 reciprocally regulate each other's mRNA with LARP1 upregulating YB1 expression, while YB-1 downregulates LARP1. This adds an extra level of complexity in the regulation of their identified mRNA targets and should be taken into account when targeting the LARP1- YB-1 complex.

The exact mechanism with which the LARP1 - YB-1 complex regulates the expression of its targets, yet remains to be defined. LARP1 is known to differentially affect the stability of its identified mRNA targets (BIK, BCL2 and mTOR) via an interaction with their 3'UTRs [327, 332] and a similar role has also been identified for YB-1 for interleukin-2 [430]. Therefore, regulation of mRNA stability could be a potential mechanism for LARP1 and YB-1 to alter the expression of their targets. However, it is possible that they are also involved in other posttranscriptional processes. Both proteins were found to relocate and interact in the nucleus upon genotoxic stress, which could indicate their involvement in splicing or the nucleocytoplasmic export of mature mRNAs (both known functions of YB-1 [395]). This warrants further investigation.

The key involvement of LARP1 in protein translation has already been recognised and it is a well-known regulator of 5'TOP mRNA homeostasis and translation. In this project, with the use of surface sensing of translation (SUnSET) assay I demonstrated that LARP1 is required

for the preservation of “de novo” protein synthesis during genotoxic stress. This suggests another potential role of LARP1 in preserving cell survival during cisplatin treatment. LARP1 could be involved in transferring mRNA transcripts (such as ATP7B or BCL2) or other “emergency” transcripts to the translational machinery to maintain cell homeostasis during the stress. It is also possible that LARP1 is itself part of the translational machinery, as it was found to interact with ribosomal proteins on an RNA independent manner.

In summary, I suggest that LARP1 is an important post-transcriptional mediator of cisplatin resistance undertaking several functions. Alongside with YB-1, (and possibly more RBPs) it is in complex with mRNA transcripts involved in pre-and post-target mechanisms of cisplatin resistance, differentially regulating their expression at a post-transcriptional level. Furthermore, LARP1 plays a key role in preserving protein translation and consequently cell survival during genotoxic stress likely via transferring “emergency” transcripts or as an active component of the translation machinery. The potential functions of LARP1 are summarised in Figure 4-1.

The multimodal manner of LARP1 involvement in mediating cisplatin resistance makes it a promising therapeutic target. This has been supported with positive outcomes from ongoing in-vivo work using RNA interference (packaged in DOPC nanoliposomes) to inhibit the expression of LARP1 and resensitise ovarian cancer xenografts to cisplatin.

Further work is required in order to identify the complete RNA interactome of LARP1 and to better dissect the exact post-transcriptional mechanisms with which LARP1 regulates the expression of its targets.

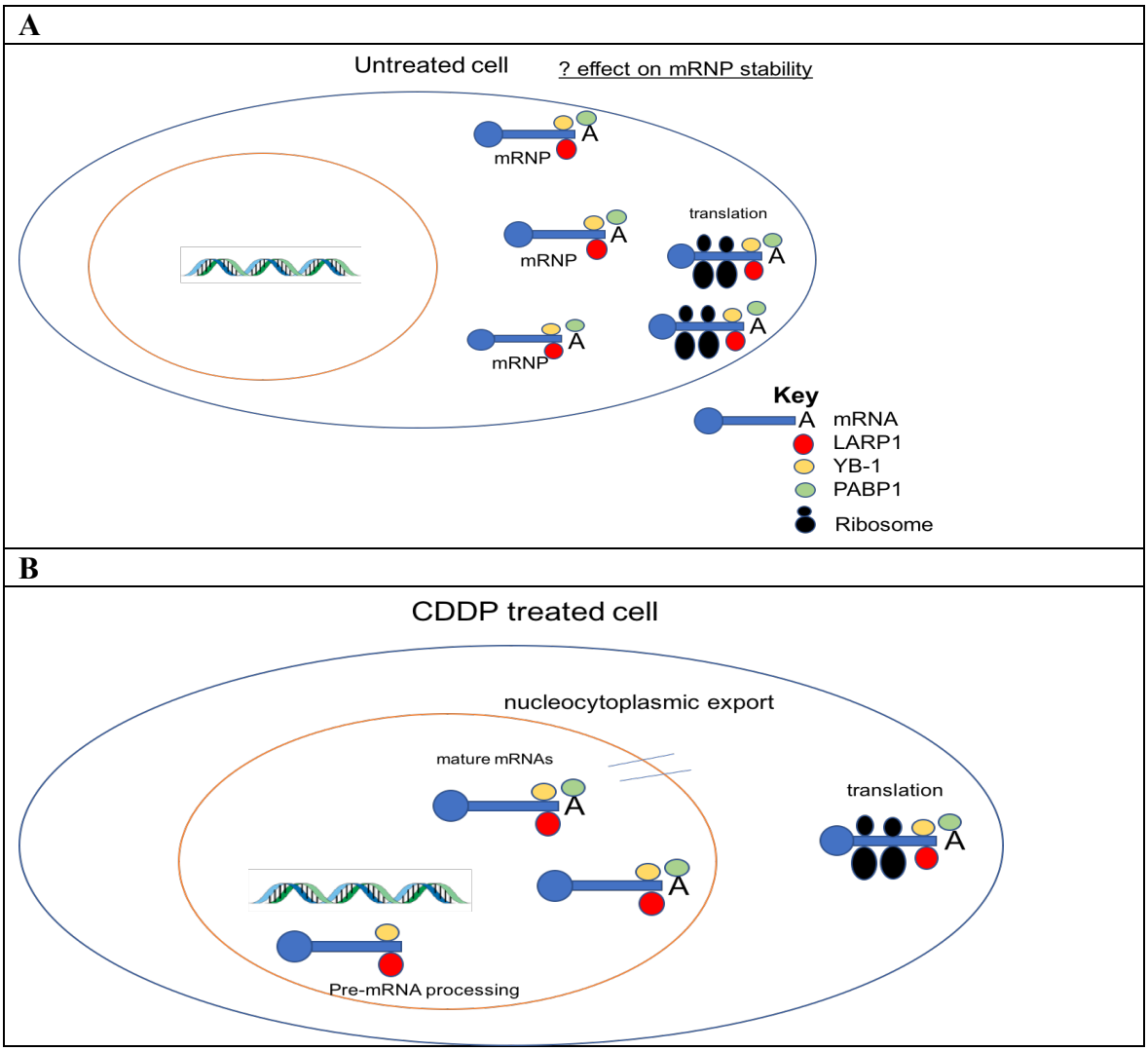


Figure 4-1 Schematic summary of the proposed cellular functions of LARP1 in the presence and absence of cisplatin in the resistant OVCAR8 cell line

In a resting state **(A)** LARP1 forms RNA-mediated cytoplasmic complexes with YB-1 and PABP1. Proteomic functional analysis implied its involvement in translation and mRNA metabolism, therefore it is likely that LARP1 interacts with YB-1 and PABP1 during mRNA translation and also as components of various mRNPs. LARP1 has been previously described to interact with the 3'UTR and 5'UTRs of its targets. (This was not investigated in the current project and the representation of the binding sites of LARP1 and YB- 1 in the schematic are arbitrary). YB-1 and LARP1 share common mRNA targets involved in mechanisms of cisplatin resistance and regulate their expression on differential manner (ATP7B expression was upregulated, while DDB2 expression downregulated). Based on previous studies on LARP1 and YB-1 this could be due an effect on the stability of these targets. Upon cisplatin treatment **(B)** the RNA mediated interaction among LARP1, YB-1 and PABP1 is maintained. LARP1 and YB-1 translocate to the nucleus whereas PABP1 remains cytoplasmic and maintains interaction with LARP1. This indicates that upon cisplatin LARP1 changes its initial protein complexes and acquires new functions to promote cell survival during genotoxic stress. Translation in the OVCAR8 cell line is maintained upon cisplatin (as proved by the SUnSET assay) and LARP1 plays an important role in its preservation. I speculated that LARP1 transclocates to the nucleus where along with YB-1 they could facilitate the nucleocytoplasmic shuttling of "emergency" mRNA transcripts to be translated for cell survival during the genotoxic stress. It is also possible that LARP1 could be directly involved in the translation of such transcripts, hence maintaining its cytoplasmic interaction with PABP1. In the nucleus LARP1 could also be involved in pre-mRNA editing, which could indicate its involvement in multiple processes of post-transcriptional regulation.

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6 APPENDICES

6.1 APPENDIX 1 - COMPREHENSIVE LISTS OF LARP1 PROTEIN INTERACTIONS IN THE OVCAR3 AND OVCAR8 CELL LINES

6.1.1 UNTREATED OVCAR 3 CELLS

Comprehensive list of the top 100 LARP1 protein interactions as identified by UHPLC-tandem mass spectrometry in the untreated OVCAR3 cells.

Accession	Gene	Score	No of significant matches	Description
P35579	MYH9	4856	156	Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4
Q8IVF2	AHNK2	3359	113	Protein AHNAK2 OS=Homo sapiens GN=AHNAK2 PE=1 SV=2
P42166	LAP2A	2420	74	Lamina-associated polypeptide 2, isoform alpha OS=Homo sapiens GN=TMPO PE=1 SV=2
Q6PKG0	LARP1	1937	85	La-related protein 1 OS=Homo sapiens GN=LARP1 PE=1 SV=2
P35580	MYH10	1883	65	Isoform 2 of Myosin-10 OS=Homo sapiens GN=MYH10
P11940	PABP1	1428	54	Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1 PE=1 SV=2
P60709	ACTB	1343	43	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1
Q13310	PABP4	1009	46	Isoform 2 of Polyadenylate-binding protein 4 OS=Homo sapiens GN=PABPC4
Q9H0A0	NAT10	783	31	N-acetyltransferase 10 OS=Homo sapiens GN=NAT10 PE=1 SV=2
H0YAS6	H0YAS6	690	19	Polyadenylate-binding protein 1 (Fragment) OS=Homo sapiens GN=PABPC1 PE=1 SV=1
P11142	HSP7C	678	32	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1
P11021	GRP78	578	21	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2
P68032	ACTC	557	28	Actin, alpha cardiac muscle 1 OS=Homo sapiens GN=ACTC1 PE=1 SV=1
F5H5D3	F5H5D3	474	14	Tubulin alpha-1C chain OS=Homo sapiens GN=TUBA1C PE=1 SV=1
Q92499	DDX1	465	23	ATP-dependent RNA helicase DDX1 OS=Homo sapiens GN=DDX1 PE=1 SV=2
Q9BYX2	TBD2A	462	17	TBC1 domain family member 2A OS=Homo sapiens GN=TBC1D2 PE=1 SV=3
Q9Y5X2	SNX8	441	15	Sorting nexin-8 OS=Homo sapiens GN=SNX8 PE=1 SV=1

P38646	GRP75	431	13	Stress-70 protein, mitochondrial OS=Homo sapiens GN=HSPA9 PE=1 SV=2
P68371	TBB4B	431	19	Tubulin beta-4B chain OS=Homo sapiens GN=TUBB4B PE=1 SV=1
Q9Y224	CN166	427	13	UPF0568 protein C14orf166 OS=Homo sapiens GN=C14orf166 PE=1 SV=1
U3KQK0	U3KQK0	421	15	Histone H2B OS=Homo sapiens GN=HIST1H2BN PE=1 SV=1
G3V4C1	G3V4C1	420	17	Heterogeneous nuclear ribonucleoproteins C1/C2 OS=Homo sapiens GN=HNRNPC PE=1 SV=1
P22234	PUR6	405	15	Isoform 2 of Multifunctional protein ADE2 OS=Homo sapiens GN=PAICS
Q9Y3I0	RTCB	402	21	tRNA-splicing ligase RtcB homolog OS=Homo sapiens GN=RTCB PE=1 SV=1
Q562R1	ACTBL	391	17	Beta-actin-like protein 2 OS=Homo sapiens GN=ACTBL2 PE=1 SV=2
P08107	HSP71	378	14	Heat shock 70 kDa protein 1A/1B OS=Homo sapiens GN=HSPA1A PE=1 SV=5
P07437	TBB5	370	18	Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2
E9PDF6	E9PDF6	370	12	Unconventional myosin-Ib OS=Homo sapiens GN=MYO1B PE=1 SV=1
P07910	HNRPC	367	17	Heterogeneous nuclear ribonucleoproteins C1/C2 OS=Homo sapiens GN=HNRNPC PE=1 SV=4
P06899	H2B1J	366	11	Histone H2B type 1-J OS=Homo sapiens GN=HIST1H2BJ PE=1 SV=3
H0YEQ8	H0YEQ8	331	12	Polyadenylate-binding protein 4 (Fragment) OS=Homo sapiens GN=PABPC4 PE=1 SV=1
P62805	H4	331	10	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2
P22626	ROA2	323	14	Isoform A2 of Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1
Q13509	TBB3	319	11	Tubulin beta-3 chain OS=Homo sapiens GN=TUBB3 PE=1 SV=2
O00425	IF2B3	308	11	Insulin-like growth factor 2 mRNA-binding protein 3 OS=Homo sapiens GN=IGF2BP3 PE=1 SV=2
P06733	ENOA	291	9	Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2
Q92900	RENT1	280	13	Isoform 2 of Regulator of nonsense transcripts 1 OS=Homo sapiens GN=UPF1
P05388	RLA0	272	10	60S acidic ribosomal protein P0 OS=Homo sapiens GN=RPLP0 PE=1 SV=1
P09651	ROA1	258	7	Isoform A1-A of Heterogeneous nuclear ribonucleoprotein A1 OS=Homo sapiens GN=HNRNPA1
J3QRS3	J3QRS3	248	11	Myosin regulatory light chain 12A OS=Homo sapiens GN=MYL12A PE=4 SV=1
Q9NR30	DDX21	220	15	Nucleolar RNA helicase 2 OS=Homo sapiens GN=DDX21 PE=1 SV=5
P62424	RL7A	215	6	60S ribosomal protein L7a OS=Homo sapiens GN=RPL7A PE=1 SV=2
Q9HAN9	NMNA1	213	11	Nicotinamide mononucleotide adenylyltransferase 1 OS=Homo sapiens GN=NMNAT1 PE=1 SV=1
P14866	HNRPL	206	9	Heterogeneous nuclear ribonucleoprotein L OS=Homo sapiens GN=HNRNPL PE=1 SV=2
P18124	RL7	202	5	60S ribosomal protein L7 OS=Homo sapiens GN=RPL7 PE=1 SV=1
Q8NEN9	PDZD8	194	8	PDZ domain-containing protein 8 OS=Homo sapiens GN=PDZD8 PE=1 SV=1

J3KTA4	J3KTA4	192	9	Probable ATP-dependent RNA helicase DDX5 OS=Homo sapiens GN=DDX5 PE=1 SV=1
Q6P2E9	EDC4	189	9	Enhancer of mRNA-decapping protein 4 OS=Homo sapiens GN=EDC4 PE=1 SV=1
B7Z6Z4	B7Z6Z4	187	7	Retinal cone rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit gamma OS=Homo sapiens GN=PDE6H PE=1 SV=1
P36578	RL4	179	7	60S ribosomal protein L4 OS=Homo sapiens GN=RPL4 PE=1 SV=5
P14618	KPYM	178	6	Pyruvate kinase PKM OS=Homo sapiens GN=PKM PE=1 SV=4
Q9Y2X7	GIT1	176	6	Isoform 3 of ARF GTPase-activating protein GIT1 OS=Homo sapiens GN=GIT1
O75367	H2AY	174	4	Isoform 1 of Core histone macro-H2A.1 OS=Homo sapiens GN=H2AFY
Q8IUE6	H2A2B	168	5	Histone H2A type 2-B OS=Homo sapiens GN=HIST2H2AB PE=1 SV=3
O00571	DDX3X	168	5	ATP-dependent RNA helicase DDX3X OS=Homo sapiens GN=DDX3X PE=1 SV=3
P08238	HS90B	164	7	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4
P01034	CYTC	159	5	Cystatin-C OS=Homo sapiens GN=CST3 PE=1 SV=1
Q9ULE6	PALD	157	4	Paladin OS=Homo sapiens GN=PALD1 PE=1 SV=3
H7BY58	H7BY58	152	4	Protein-L-isoaspartate O-methyltransferase OS=Homo sapiens GN=PCMT1 PE=1 SV=1
P0C0S8	H2A1	151	5	Histone H2A type 1 OS=Homo sapiens GN=HIST1H2AG PE=1 SV=2
P12035	K2C3	150	8	Keratin, type II cytoskeletal 3 OS=Homo sapiens GN=KRT3 PE=1 SV=3
P04406	G3P	149	9	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3
P39023	RL3	146	6	60S ribosomal protein L3 OS=Homo sapiens GN=RPL3 PE=1 SV=2
Q5VU59	Q5VU59	145	3	Uncharacterized protein OS=Homo sapiens GN=TPM3 PE=1 SV=1
P38159	RBMX	138	6	RNA-binding motif protein, X chromosome OS=Homo sapiens GN=RBMX PE=1 SV=3
P52597	HNRPF	133	3	Heterogeneous nuclear ribonucleoprotein F OS=Homo sapiens GN=HNRNPF PE=1 SV=3
O43809	CPSF5	130	6	Cleavage and polyadenylation specificity factor subunit 5 OS=Homo sapiens GN=NUDT21 PE=1 SV=1
P23396	RS3	127	7	40S ribosomal protein S3 OS=Homo sapiens GN=RPS3 PE=1 SV=2
P06396	GELS	123	4	Isoform 2 of Gelsolin OS=Homo sapiens GN=GSN
Q14161	GIT2	122	5	Isoform 10 of ARF GTPase-activating protein GIT2 OS=Homo sapiens GN=GIT2
P61981	1433G	122	5	14-3-3 protein gamma OS=Homo sapiens GN=YWHAG PE=1 SV=2
P31942	HNRH3	122	2	Isoform 2 of Heterogeneous nuclear ribonucleoprotein H3 OS=Homo sapiens GN=HNRNPH3
Q9UM54	MYO6	115	4	Isoform 2 of Unconventional myosin-VI OS=Homo sapiens GN=MYO6
P27348	1433T	111	5	14-3-3 protein theta OS=Homo sapiens GN=YWHAQ PE=1 SV=1
M0R0F0	M0R0F0	110	2	40S ribosomal protein S5 (Fragment) OS=Homo sapiens GN=RPS5 PE=1 SV=1
J3KTE4	J3KTE4	108	2	Ribosomal protein L19 OS=Homo sapiens GN=RPL19 PE=1 SV=1

G5EA36	G5EA36	107	4	Cell division cycle 27, isoform CRA_c OS=Homo sapiens GN=CDC27 PE=1 SV=1
P52272	HNRPM	103	3	Heterogeneous nuclear ribonucleoprotein M OS=Homo sapiens GN=HNRNPM PE=1 SV=3
C9JNW5	C9JNW5	100	4	60S ribosomal protein L24 OS=Homo sapiens GN=RPL24 PE=1 SV=1
X1WI28	X1WI28	100	6	60S ribosomal protein L10 (Fragment) OS=Homo sapiens GN=RPL10 PE=1 SV=1
F5H2Z3	F5H2Z3	99	3	Polyubiquitin-C (Fragment) OS=Homo sapiens GN=UBC PE=4 SV=1
P51991	ROA3	98	3	Heterogeneous nuclear ribonucleoprotein A3 OS=Homo sapiens GN=HNRNPA3 PE=1 SV=2
P30048	PRDX3	97	4	Isoform 2 of Thioredoxin-dependent peroxide reductase, mitochondrial OS=Homo sapiens GN=PRDX3
P22087	FBRL	96	5	rRNA 2'-O-methyltransferase fibrillarin OS=Homo sapiens GN=FBL PE=1 SV=2
Q9HAU5	RENT2	95	4	Regulator of nonsense transcripts 2 OS=Homo sapiens GN=UPF2 PE=1 SV=1
P04083	ANXA1	95	3	Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2
P62263	RS14	95	2	40S ribosomal protein S14 OS=Homo sapiens GN=RPS14 PE=1 SV=3
P62854	RS26	93	2	40S ribosomal protein S26 OS=Homo sapiens GN=RPS26 PE=1 SV=3
O75531	BAF	92	4	Barrier-to-autointegration factor OS=Homo sapiens GN=BANF1 PE=1 SV=1
Q9Y4B5	MTCL1	92	3	Microtubule cross-linking factor 1 OS=Homo sapiens GN=MTCL1 PE=1 SV=5
P31946	1433B	87	5	Isoform Short of 14-3-3 protein beta/alpha OS=Homo sapiens GN=YWHAB
Q9NZB2	F120A	87	1	Isoform F of Constitutive coactivator of PPAR-gamma-like protein 1 OS=Homo sapiens GN=FAM120A
P62277	RS13	87	4	40S ribosomal protein S13 OS=Homo sapiens GN=RPS13 PE=1 SV=2
D6R9P3	D6R9P3	87	2	Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB PE=1 SV=1
P62753	RS6	87	2	40S ribosomal protein S6 OS=Homo sapiens GN=RPS6 PE=1 SV=1
Q08211	DHX9	84	2	ATP-dependent RNA helicase A OS=Homo sapiens GN=DHX9 PE=1 SV=4
Q5T4L4	Q5T4L4	84	3	40S ribosomal protein S27 OS=Homo sapiens GN=RPS27 PE=1 SV=1
G8JLB6	G8JLB6	84	2	Heterogeneous nuclear ribonucleoprotein H OS=Homo sapiens GN=HNRNPH1 PE=1 SV=1
P62995	TRA2B	83	3	Transformer-2 protein homolog beta OS=Homo sapiens GN=TRA2B PE=1 SV=1

6.1.2 CISPLATIN TREATED OVCAR3 CELLS

Comprehensive list of LARP1 protein interactors as identified by by UHPLC- tandem mass spectrometry in the cisplatin treated OVCAR3 cells.

Accession	Gene	Score	No of significant matches	Description
Q8IVF2	AHNK2	1592	63	Isoform 3 of Protein AHNAK2 OS=Homo sapiens GN=AHNAK2
P42166	LAP2A	849	34	Lamina-associated polypeptide 2, isoform alpha OS=Homo sapiens GN=TMPO PE=1 SV=2
Q9H0A0	NAT10	435	25	N-acetyltransferase 10 OS=Homo sapiens GN=NAT10 PE=1 SV=2
P22234	PUR6	312	12	Isoform 2 of Multifunctional protein ADE2 OS=Homo sapiens GN=PAICS
F5H5D3	F5H5D3	301	8	Tubulin alpha-1C chain OS=Homo sapiens GN=TUBA1C PE=1 SV=1
Q6PKG0	LARP1	294	19	La-related protein 1 OS=Homo sapiens GN=LARP1 PE=1 SV=2
Q9HAN9	NMNA1	229	11	Nicotinamide mononucleotide adenylyltransferase 1 OS=Homo sapiens GN=NMNAT1 PE=1 SV=1
P08107	HSP71	217	9	Isoform 2 of Heat shock 70 kDa protein 1A/1B OS=Homo sapiens GN=HSPA1A
Q14155	ARHG7	126	5	Isoform 1 of Rho guanine nucleotide exchange factor 7 OS=Homo sapiens GN=ARHGEF7
Q6P2E9	EDC4	126	6	Enhancer of mRNA-decapping protein 4 OS=Homo sapiens GN=EDC4 PE=1 SV=1
O00425	IF2B3	126	4	Insulin-like growth factor 2 mRNA-binding protein 3 OS=Homo sapiens GN=IGF2BP3 PE=1 SV=2
Q00839	HNRPU	117	4	Isoform Short of Heterogeneous nuclear ribonucleoprotein U OS=Homo sapiens GN=HNRNPU
P11940	PABP1	113	3	Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1 PE=1 SV=2
P01602	KV110	85	2	Ig kappa chain V-I region HK102 (Fragment) OS=Homo sapiens GN=IGKV1-5 PE=4 SV=1
U3KQK0	U3KQK0	69	2	Histone H2B OS=Homo sapiens GN=HIST1H2BN PE=1 SV=1
P23246	SFPQ	69	4	Splicing factor, proline- and glutamine-rich OS=Homo sapiens GN=SFPQ PE=1 SV=2
P06899	H2B1J	68	2	Histone H2B type 1-J OS=Homo sapiens GN=HIST1H2BJ PE=1 SV=3
Q92499	DDX1	65	2	Isoform 3 of ATP-dependent RNA helicase DDX1 OS=Homo sapiens GN=DDX1
P02747	C1QC	63	1	Complement C1q subcomponent subunit C OS=Homo sapiens GN=C1QC PE=1 SV=3
P01023	A2MG	60	2	Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3
P04637	P53	59	1	Isoform 4 of Cellular tumor antigen p53 OS=Homo sapiens GN=TP53
E9PB61	E9PB61	58	4	THO complex subunit 4 OS=Homo sapiens GN=ALYREF PE=1 SV=1
E9PL09	E9PL09	57	1	40S ribosomal protein S3 OS=Homo sapiens GN=RPS3 PE=1 SV=1
Q8NEN9	PDZD8	55	1	PDZ domain-containing protein 8 OS=Homo sapiens

				GN=PDZD8 PE=1 SV=1
Q9Y2X7	GIT1	55	3	Isoform 3 of ARF GTPase-activating protein GIT1 OS=Homo sapiens GN=GIT1
Q02413	DSG1	55	1	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2
Q9H2G2	SLK	53	1	STE20-like serine/threonine-protein kinase OS=Homo sapiens GN=SLK PE=1 SV=1
M0R0F0	M0R0F0	53	1	40S ribosomal protein S5 (Fragment) OS=Homo sapiens GN=RPS5 PE=1 SV=1
H3BN98	H3BN98	53	1	Uncharacterized protein (Fragment) OS=Homo sapiens PE=4 SV=2
B7Z972	B7Z972	51	1	Protein-L-isoaspartate O-methyltransferase OS=Homo sapiens GN=PCMT1 PE=1 SV=1
P62753	RS6	49	1	40S ribosomal protein S6 OS=Homo sapiens GN=RPS6 PE=1 SV=1
O60812	HNRC1	49	1	Heterogeneous nuclear ribonucleoprotein C-like 1 OS=Homo sapiens GN=HNRNPCL1 PE=1 SV=1
Q6NUK1	SCMC1	48	1	Isoform 2 of Calcium-binding mitochondrial carrier protein SCaMC-1 OS=Homo sapiens GN=SLC25A24
A0A075B6P5	A0A075B6P5	48	1	Protein IGKV2D-28 (Fragment) OS=Homo sapiens GN=IGKV2D-28 PE=4 SV=1
B5MCP9	B5MCP9	48	1	40S ribosomal protein S7 OS=Homo sapiens GN=RPS7 PE=1 SV=1
K7EMH1	K7EMH1	47	2	60S ribosomal protein L22 (Fragment) OS=Homo sapiens GN=RPL22 PE=1 SV=1
K7ES89	K7ES89	45	1	Dual-specificity protein phosphatase 3 (Fragment) OS=Homo sapiens GN=DUSP3 PE=1 SV=1
P25311	ZA2G	44	1	Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=1 SV=2
H7BY58	H7BY58	44	1	Protein-L-isoaspartate O-methyltransferase OS=Homo sapiens GN=PCMT1 PE=1 SV=1
H0Y6E7	H0Y6E7	44	2	RNA-binding motif protein, X chromosome, N-terminally processed (Fragment) OS=Homo sapiens GN=RBMX PE=1 SV=2
H3BM89	H3BM89	43	1	60S ribosomal protein L4 OS=Homo sapiens GN=RPL4 PE=1 SV=1
P07900	HS90A	43	1	Isoform 2 of Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1
Q9NYL2	MLTK	43	1	Mitogen-activated protein kinase kinase kinase MLT OS=Homo sapiens GN=ZAK PE=1 SV=3
M0R210	M0R210	43	1	40S ribosomal protein S16 OS=Homo sapiens GN=RPS16 PE=1 SV=1
Q9H967	WDR76	42	1	WD repeat-containing protein 76 OS=Homo sapiens GN=WDR76 PE=1 SV=2
B4DS13	B4DS13	42	1	Eukaryotic translation initiation factor 4B OS=Homo sapiens GN=EIF4B PE=1 SV=1
Q2M389	WASH7	41	1	WASH complex subunit 7 OS=Homo sapiens GN=KIAA1033 PE=1 SV=2
Q5JR95	Q5JR95	41	1	40S ribosomal protein S8 OS=Homo sapiens GN=RPS8 PE=1 SV=1
Q9UKN7	MYO15	41	1	Unconventional myosin-XV OS=Homo sapiens GN=MYO15A PE=1 SV=2
P01857	IGHG1	41	2	Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1
J3KTE4	J3KTE4	39	1	Ribosomal protein L19 OS=Homo sapiens GN=RPL19 PE=1 SV=1
F8WJN3	F8WJN3	37	1	Cleavage and polyadenylation-specificity factor subunit 6 OS=Homo sapiens GN=CPSF6 PE=1 SV=1
Q9BTM1	H2AJ	36	1	Histone H2A.J OS=Homo sapiens GN=H2AFJ PE=1 SV=1

P62829	RL23	35	1	60S ribosomal protein L23 OS=Homo sapiens GN=RPL23 PE=1 SV=1
P11940	PABP1	33	1	Isoform 2 of Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1
Q00839	HNRPU	32	1	Heterogeneous nuclear ribonucleoprotein U OS=Homo sapiens GN=HNRNPU PE=1 SV=6
P62266	RS23	29	2	40S ribosomal protein S23 OS=Homo sapiens GN=RPS23 PE=1 SV=3

6.1.3 UNTREATED OVCAR 8 CELLS

Comprehensive list of the top 100 LARP1 protein interactions as identified by UHPLC-tandem mass spectrometry in the untreated OVCAR8 cells.

Accession	Gene	Score	No of significant matches	Description
P42166	LAP2A	1366	49	Lamina-associated polypeptide 2, isoform alpha OS
Q8IVF2	AHNK2	1005	44	Isoform 3 of Protein AHNAK2 OS
P11940	PABP1	860	39	Polyadenylate-binding protein 1 OS
Q6PKG0	LARP1	647	43	La-related protein 1 OS
P46821	MAP1B	370	15	Microtubule-associated protein 1B OS
P67809	YBOX1	297	10	Nuclease-sensitive element-binding protein 1 OS
Q13310	PABP4	287	15	Isoform 2 of Polyadenylate-binding protein 4 OS
Q9BYX2	TBD2A	274	14	TBC1 domain family member 2A OS
Q9HCE1	MOV10	245	9	Putative helicase MOV-10 OS
Q9Y2T7	YBOX2	217	6	Y-box-binding protein 2 OS
A8MXP9	A8MXP9	188	10	Matrin-3 OS
G3V2Q1	G3V2Q1	186	6	Heterogeneous nuclear ribonucleoproteins C1/C2 OS
P16989	YBOX3	168	7	Isoform 2 of Y-box-binding protein 3 OS
Q92900	RENT1	168	11	Isoform 2 of Regulator of nonsense transcripts 1 OS
P46013	KI67	162	9	Antigen KI-67 OS
P52272	HNRPM	156	7	Isoform 2 of Heterogeneous nuclear ribonucleoprotein M OS
Q9Y623	MYH4	154	7	Myosin-4 OS
O60506	HNRPQ	153	7	Isoform 2 of Heterogeneous nuclear ribonucleoprotein Q OS
P22626	ROA2	131	6	Heterogeneous nuclear ribonucleoproteins A2/B1 OS
Q00839	HNRPU	129	6	Heterogeneous nuclear ribonucleoprotein U OS
E9PBS1	E9PBS1	121	5	Phosphoribosylaminoimidazole carboxylase (Fragment) OS
P09651	ROA1	120	5	Isoform A1-A of Heterogeneous nuclear ribonucleoprotein A1 OS
O95714	HERC2	119	11	E3 ubiquitin-protein ligase HERC2 OS
Q5SW79	CE170	118	7	Centrosomal protein of 170 kDa OS
Q9Y4B5	MTCL1	117	6	Microtubule cross-linking factor 1 OS

B3KS98	B3KS98	115	1	Eukaryotic translation initiation factor 3 subunit H OS
J3QRU8	J3QRU8	113	1	ARF GTPase-activating protein GIT1 OS
P04406	G3P	109	4	Glyceraldehyde-3-phosphate dehydrogenase OS
P06733	ENOA	108	2	Isoform MBP-1 of Alpha-enolase OS
Q7L7L0	H2A3	108	1	Histone H2A type 3 OS
O43390	HNRPR	107	4	Isoform 2 of Heterogeneous nuclear ribonucleoprotein R OS
Q9NZI8	IF2B1	106	2	Insulin-like growth factor 2 mRNA-binding protein 1 OS
B1ALK7	B1ALK7	102	6	Rho guanine nucleotide exchange factor 7 OS
P06899	H2B1J	99	3	Histone H2B type 1-J OS
R4GNG3	R4GNG3	98	3	ARF GTPase-activating protein GIT2 (Fragment) OS
H3BLZ8	H3BLZ8	98	3	Probable ATP-dependent RNA helicase DDX17 OS
J3QS41	J3QS41	94	3	Probable helicase with zinc finger domain OS
P46781	RS9	91	1	40S ribosomal protein S9 OS
D6RAT0	D6RAT0	90	4	40S ribosomal protein S3a OS
E7ETY2	E7ETY2	89	3	Treacle protein OS
D6R9P3	D6R9P3	87	2	Heterogeneous nuclear ribonucleoprotein A/B OS
P08670	VIME	86	3	Vimentin OS
E7EQV3	E7EQV3	86	2	Polyadenylate-binding protein 1 OS
P23396	RS3	85	4	40S ribosomal protein S3 OS
P05089	ARGI1	85	3	Isoform 2 of Arginase-1 OS
J3KNF5	J3KNF5	82	5	Centrosomal protein of 290 kDa OS
Q15424	SAFB1	80	3	Isoform 2 of Scaffold attachment factor B1 OS
Q08554	DSC1	80	3	Desmocollin-1 OS
P62857	RS28	79	3	40S ribosomal protein S28 OS
P61247	RS3A	79	3	40S ribosomal protein S3a OS
B0QY89	B0QY89	76	2	Eukaryotic translation initiation factor 3 subunit L OS
P01023	A2MG	76	3	Alpha-2-macroglobulin OS
P55884	EIF3B	75	1	Isoform 2 of Eukaryotic translation initiation factor 3 subunit B OS
Q93077	H2A1C	75	2	Histone H2A type 1-C OS
P01602	KV110	75	1	Ig kappa chain V-I region HK102 (Fragment) OS
P14866	HNRPL	73	4	Heterogeneous nuclear ribonucleoprotein L OS
Q5VVC8	Q5VVC8	73	1	60S ribosomal protein L11 (Fragment) OS
A6NMY6	AXA2L	73	2	Putative annexin A2-like protein OS
Q7Z4S6	KI21A	73	2	Isoform 3 of Kinesin-like protein KIF21A OS
Q5JP53	Q5JP53	72	2	Tubulin beta chain OS
K7EJV9	K7EJV9	72	2	60S ribosomal protein L23a (Fragment) OS
J3QSB4	J3QSB4	71	2	60S ribosomal protein L13 (Fragment) OS
Q68EM7	RHG17	71	3	Rho GTPase-activating protein 17 OS
Q9H0A0	NAT10	71	4	N-acetyltransferase 10 OS
F8WB72	F8WB72	70	1	60S ribosomal protein L35a OS
I3L3P7	I3L3P7	70	2	40S ribosomal protein S15a OS
P62258	1433E	69	2	14-3-3 protein epsilon OS

B1AN99	B1AN99	69	1	Trypsin-3 (Fragment) OS
C9JNW5	C9JNW5	68	1	60S ribosomal protein L24 OS
B7Z4C8	B7Z4C8	67	1	60S ribosomal protein L31 OS
O00571	DDX3X	67	1	Isoform 2 of ATP-dependent RNA helicase DDX3X OS
E9PJD9	E9PJD9	67	2	60S ribosomal protein L27a OS
Q5D862	FILA2	66	1	Filaggrin-2 OS
P62995	TRA2B	66	2	Isoform 3 of Transformer-2 protein homolog beta OS
P26373	RL13	65	2	60S ribosomal protein L13 OS
P62424	RL7A	64	2	60S ribosomal protein L7a OS
O00303	EIF3F	63	2	Eukaryotic translation initiation factor 3 subunit F OS
B0YJC4	B0YJC4	63	1	Vimentin OS
P31942	HNRH3	63	2	Isoform 2 of Heterogeneous nuclear ribonucleoprotein H3 OS
Q12906	ILF3	62	3	Isoform 7 of Interleukin enhancer-binding factor 3 OS
P17844	DDX5	61	3	Isoform 2 of Probable ATP-dependent RNA helicase DDX5 OS
P49792	RBP2	61	1	E3 SUMO-protein ligase RanBP2 OS
E9PLL6	E9PLL6	61	2	60S ribosomal protein L27a OS
P0CW22	RS17L	61	1	40S ribosomal protein S17-like OS
P29508	SPB3	61	1	Serpin B3 OS
Q9HAN9	NMNA1	60	2	Nicotinamide mononucleotide adenylyltransferase 1 OS
I7HJJ0	I7HJJ0	60	1	ADP/ATP translocase 3 (Fragment) OS
J3QS39	J3QS39	60	2	Ubiquitin (Fragment) OS
D6REM6	D6REM6	59	3	Matrin-3 OS
M0R3F6	M0R3F6	59	1	SURP and G-patch domain-containing protein 2 OS
P04040	CATA	58	1	Catalase OS
Q5JR04	Q5JR04	57	2	Mov10, Moloney leukemia virus 10, homolog (Mouse), isoform CRA_a OS
P31944	CASPE	57	1	Caspase-14 OS
P25311	ZA2G	57	1	Zinc-alpha-2-glycoprotein OS
C9JZG1	C9JZG1	57	1	Eukaryotic translation initiation factor 3 subunit B (Fragment) OS
Q86TI0	TBCD1	57	1	Isoform 2 of TBC1 domain family member 1 OS
Q13136	LIPA1	57	3	Isoform 2 of Liprin-alpha-1 OS
H0YB22	H0YB22	56	1	40S ribosomal protein S14 (Fragment) OS

6.1.4 CISPLATIN TREATED OVCAR 8 CELLS

Comprehensive list of the top 100 LARP1 protein interactions as identified by UHPLC-tandem mass spectrometry in the untreated OVCAR8 cells.

Accession	Gene	Score	No of significant matches	Description
P42166	LAP2A	1687	74	Lamina-associated polypeptide 2, isoform alpha OS
Q6PKG0	LARP1	1562	77	La-related protein 1 OS
P11940	PABP1	1298	61	Polyadenylate-binding protein 1 OS
Q8IVF2	AHNK2	1250	52	Isoform 3 of Protein AHNAK2 OS
Q9Y4B5	MTCL1	1105	48	Microtubule cross-linking factor 1 OS
Q5SW79	CE170	984	42	Centrosomal protein of 170 kDa OS
Q13310	PABP4	850	39	Polyadenylate-binding protein 4
P67809	YBOX1	492	13	Nuclease-sensitive element-binding protein 1 OS
Q9Y5X2	SNX8	436	14	Sorting nexin-8 OS
Q14315	FLNC	385	16	Isoform 2 of Filamin-C OS
F5H5D3	F5H5D3	334	12	Tubulin alpha-1C chain OS
P07437	TBB5	320	13	Tubulin beta chain OS
Q5JR04	Q5JR04	315	14	Mov10, Moloney leukemia virus 10, homolog (Mouse), isoform CRA_a OS
A8MXP9	A8MXP9	290	13	Matrin-3 OS
P08238	HS90B	275	10	Heat shock protein HSP 90-beta OS
P14866	HNRPL	268	7	Heterogeneous nuclear ribonucleoprotein L OS
Q9Y2T7	YBOX2	257	7	Y-box-binding protein 2 OS
Q92499	DDX1	237	12	ATP-dependent RNA helicase DDX1 OS
Q9H0A0	NAT10	229	9	N-acetyltransferase 10 OS
Q14155	ARHG7	219	7	Isoform 1 of Rho guanine nucleotide exchange factor 7 OS
Q9ULE6	PALD	219	6	Paladin OS
P16989	YBOX3	204	6	Isoform 2 of Y-box-binding protein 3 OS
P52272	HNRPM	201	12	Isoform 2 of Heterogeneous nuclear ribonucleoprotein M OS
Q92900	RENT1	197	11	Isoform 2 of Regulator of nonsense transcripts 1 OS
P61978	HNRPK	197	5	Isoform 2 of Heterogeneous nuclear ribonucleoprotein K OS
Q8NCA5	FA98A	192	5	Isoform 2 of Protein FAM98A OS
Q68EM7	RHG17	184	8	Rho GTPase-activating protein 17 OS
G3V4C1	G3V4C1	182	9	Heterogeneous nuclear ribonucleoproteins C1/C2 OS
P62736	ACTA	173	9	Actin, aortic smooth muscle OS
Q659C4	LAR1B	170	6	Isoform 2 of La-related protein 1B OS
Q6P2E9	EDC4	163	9	Enhancer of mRNA-decapping protein 4 OS
Q00839	HNRPU	159	6	Heterogeneous nuclear ribonucleoprotein U OS
Q9BUF5	TBB6	157	7	Tubulin beta-6 chain OS

G8JLB6	G8JLB6	145	4	Heterogeneous nuclear ribonucleoprotein H OS
Q9Y623	MYH4	142	8	Myosin-4 OS
Q9Y3I0	RTCB	138	7	tRNA-splicing ligase RtcB homolog OS
B1ALK7	B1ALK7	131	5	Rho guanine nucleotide exchange factor 7 OS
J3KTA4	J3KTA4	126	6	Probable ATP-dependent RNA helicase DDX5 OS
B4E1K0	B4E1K0	125	3	Kinesin-like protein KIF23 OS
Q5T8M8	Q5T8M8	120	7	Actin, alpha skeletal muscle OS
P14625	ENPL	117	3	Endoplasmin OS
P61981	1433G	113	6	14-3-3 protein gamma OS
P46781	RS9	111	2	40S ribosomal protein S9 OS
E9PBS1	E9PBS1	111	6	Phosphoribosylaminoimidazole carboxylase (Fragment) OS
O60814	H2B1K	109	3	Histone H2B type 1-K OS
P05783	K1C18	108	2	Keratin, type I cytoskeletal 18 OS
Q5BKZ1	ZN326	105	5	DBIRD complex subunit ZNF326 OS
O00571	DDX3X	104	4	Isoform 2 of ATP-dependent RNA helicase DDX3X OS
P13535	MYH8	102	4	Myosin-8 OS
P31946	1433B	99	4	Isoform Short of 14-3-3 protein beta/alpha OS
Q07065	CKAP4	97	2	Cytoskeleton-associated protein 4 OS
J3QRU8	J3QRU8	94	3	ARF GTPase-activating protein GIT1 OS
A8MUD9	A8MUD9	93	2	60S ribosomal protein L7 OS
H7C1J8	H7C1J8	91	1	Heterogeneous nuclear ribonucleoprotein A3 (Fragment) OS
P10809	CH60	87	3	60 kDa heat shock protein, mitochondrial OS
P27348	1433T	87	3	14-3-3 protein theta OS
P13639	EF2	85	2	Elongation factor 2 OS
H0YEN5	H0YEN5	85	3	40S ribosomal protein S2 (Fragment) OS
A6NMY6	AXA2L	85	2	Putative annexin A2-like protein OS
Q9NZB2	F120A	84	2	Isoform F of Constitutive coactivator of PPAR-gamma-like protein 1 OS
D6R9P3	D6R9P3	84	3	Heterogeneous nuclear ribonucleoprotein A/B OS
Q8N371	KDM8	84	2	Isoform 3 of Lysine-specific demethylase 8 OS
O43390	HNRPR	78	2	Isoform 2 of Heterogeneous nuclear ribonucleoprotein R OS
P51991	ROA3	78	1	Isoform 2 of Heterogeneous nuclear ribonucleoprotein A3 OS
Q14161	GIT2	76	5	Isoform 11 of ARF GTPase-activating protein GIT2 OS
P04406	G3P	76	1	Glyceraldehyde-3-phosphate dehydrogenase OS
B1AN62	B1AN62	76	1	Nicotinamide mononucleotide adenylyltransferase 1 OS
P39019	RS19	76	1	40S ribosomal protein S19 OS
Q3MHD2	LSM12	76	1	Isoform 2 of Protein LSM12 homolog OS
P62258	1433E	75	3	14-3-3 protein epsilon OS
H0YKX5	H0YKX5	73	1	Tropomyosin alpha-1 chain (Fragment) OS
P01602	KV110	72	1	Ig kappa chain V-I region HK102 (Fragment) OS
D6RG13	D6RG13	71	2	40S ribosomal protein S3a (Fragment) OS
P31942	HNRH3	70	1	Isoform 2 of Heterogeneous nuclear ribonucleoprotein H3 OS
K7EJV9	K7EJV9	70	1	60S ribosomal protein L23a (Fragment) OS
Q5T6W5	Q5T6W5	70	4	Heterogeneous nuclear ribonucleoprotein K OS

J3QSB4	J3QSB4	69	2	60S ribosomal protein L13 (Fragment) OS
P0C0S8	H2A1	69	1	Histone H2A type 1 OS
F8WJN3	F8WJN3	68	2	Cleavage and polyadenylation-specificity factor subunit 6 OS
Q9NR30	DDX21	68	1	Isoform 2 of Nucleolar RNA helicase 2 OS
C9J3N8	C9J3N8	68	1	Heat shock protein beta-1 OS
Q96A32	MLRS	68	1	Myosin regulatory light chain 2, skeletal muscle isoform OS
Q9H0H5	RGAP1	67	1	Rac GTPase-activating protein 1 OS
J3QKY4	J3QKY4	66	2	Proline-rich protein 11 (Fragment) OS
Q9Y6X9	MORC2	65	2	MORC family CW-type zinc finger protein 2 OS
P0CW22	RS17L	65	1	40S ribosomal protein S17-like OS
P38159	RBMX	64	2	RNA-binding motif protein, X chromosome OS
P55795	HNRH2	64	2	Heterogeneous nuclear ribonucleoprotein H2 OS
P36578	RL4	63	2	60S ribosomal protein L4 OS
P39023	RL3	63	2	60S ribosomal protein L3 OS
Q9H8Y8	GORS2	63	1	Golgi reassembly-stacking protein 2 OS
J3KTE4	J3KTE4	63	1	Ribosomal protein L19 OS
P06733	ENOA	62	1	Isoform MBP-1 of Alpha-enolase OS
B0QY89	B0QY89	62	1	Eukaryotic translation initiation factor 3 subunit L OS
K7EKS7	K7EKS7	62	1	60S ribosomal protein L22 OS
E9PPJ0	E9PPJ0	61	2	Splicing factor 3B subunit 2 OS
Q12905	ILF2	61	2	Interleukin enhancer-binding factor 2 OS
P05141	ADT2	60	2	ADP/ATP translocase 2 OS
P62979	RS27A	60	1	Ubiquitin-40S ribosomal protein S27a OS

6.2 VIABILITY AND APOPTOSIS ASSAYS

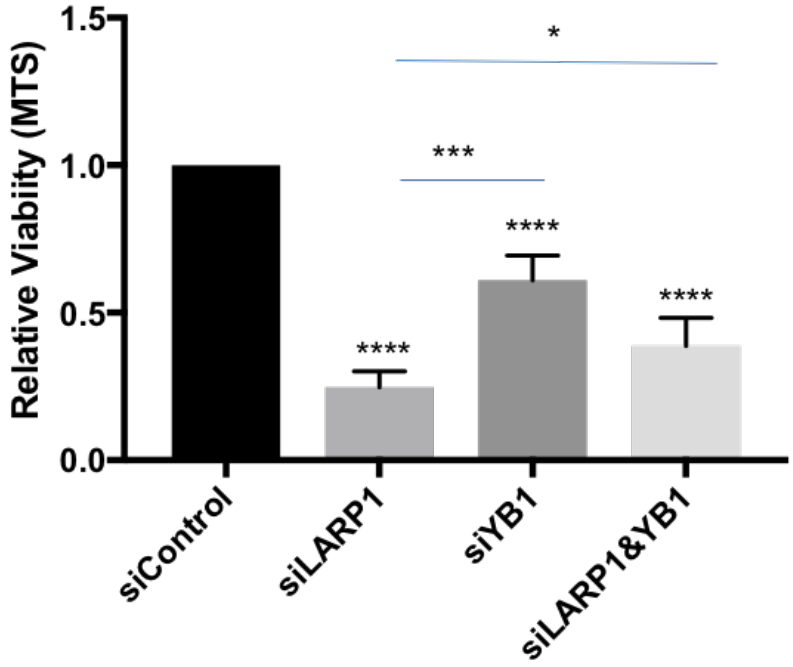

Figure 6-1 Combination of cisplatin and LARP1 knockdown results in greater decrease in viability compared to YB-1 or double knockdown in the resistant OVCAR8 cells.

Normalised cell viability determined by MTS assay in OVCAR8 cells undergoing siRNA mediated transient LARP1, YB-1 or double knockdown (48h) followed by cisplatin (25μM for 48h).

Student t-test ****p<0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Minimum of three experimental repeats. Error bars represent SEM.

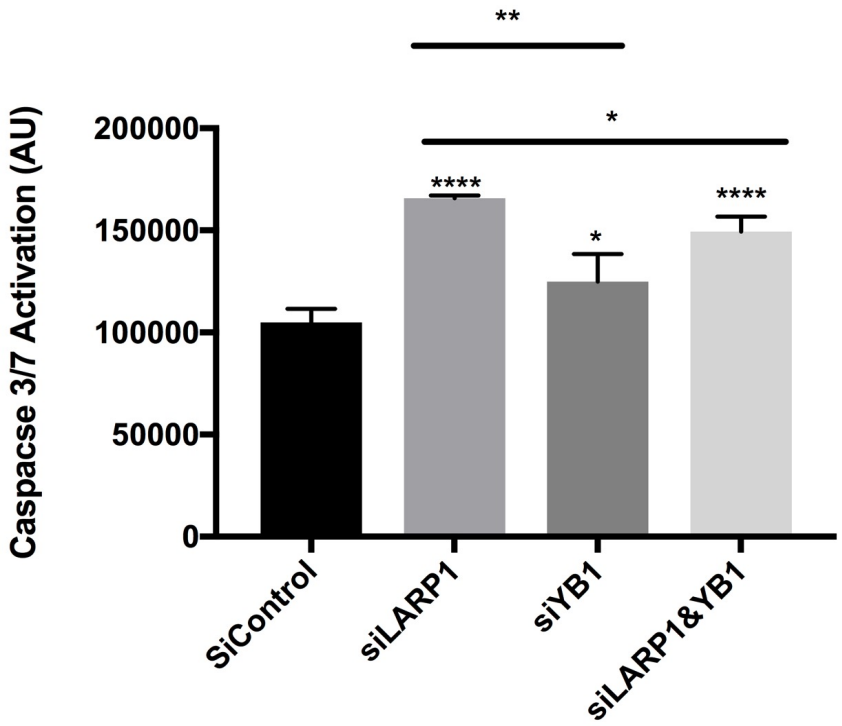

Figure 6-2 Combination of cisplatin and LARP1 knockdown results in greater apoptosis compared to YB-1 or double knockdown in the resistant OVCAR8 cell line.

Levels of cleaved Caspase 3/7 were determined by the CaspaseGlo assay in OVCAR8 cell line upon transient siRNA- mediated LAR1, YB-1 or double knock-down (48h) followed by treatment with cisplatin (25μM for 48h).

Student t-test ****p<0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Minimum of three experimental repeats. Error bars represent SEM.

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