Imperial College London | ICR The Institute of Cancer Research

Proposal Title: Investigating the roles of the proteasome vs. autophagy, two major protein degradation pathways, in degradation and stabilisation of the proteome of ovarian high-grade serous carcinoma (HGSC)

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Proposal outline

Project rationale and objectives

High-grade serous carcinoma (HGSC) of ovaries is a high-unmet-need disease in women worldwide. Tumours are characterised by frequent mutations in TP53 and BRCA1/2 and high prevalence of copy number alterations (CNAs)[1]. The high degree of genomic instability observed in HGSC, and exploited therapeutically by the recently developed PARP inhibitors, translates into a perturbed and varied proteome, with several groups developing proteome-based signatures to predict the disease outcome[2-4]. Notwithstanding the link between genomic instability and the global proteome changes, it remains unclear how this highly perturbed proteome is stabilised in HGSC cells. The intimate interplay between the mRNA translation, chaperone-mediated protein folding and protein degradation machineries determines the proteome stability. Two major cellular pathways are responsible for protein degradation: the ubiquitin-proteasome system (UPS) and the lysosome-autophagy pathway (autophagy). Whereas the UPS relies on a multi-subunit barrel-shaped proteolytic machine, which degrades single polypeptide chains, autophagy is a membrane-based system that sequesters and removes large protein complexes and protein aggregates[5]. By manipulating the proteasomal and the autophagic activity in HGSC cells, we will address the question: what relative contribution does the UPS vs. autophagy each have in degrading/stabilising the proteome in HGSC? By revealing the nodes/targets in the protein networks and understanding mechanisms of the proteome stabilisation via the UPS/autophagy, we will be able to propose new therapeutic strategies to tackle this deadly disease.

Researh plan

1. A panel of HGSC cell lines is available (e.g., OVCAR4, **Fig. 1C**, OVSAHO, COV362). These will be transduced with doxycycline (dox)-inducible CRISPR/dCas9-KRAB constructs that target

genes essential for the proteasome (e.g., PSMD4) or autophagy (e.g., ATG7, BECN1, ATG4B). The advantage of this gene-targeting system is in that gene expression can be reversibly inactivated by the dox-inducible expression of the catalytically inactive dCas9 fused with the KRAB transcriptional repressor, which is targeted to the gene promoter using sgRNA[6]. This system allows modulation of gene expression both *in vitro* (**Fig. 1B**) and *in vivo*. The engineered HGSC cells will be characterised in terms of the target modulation (gene and protein expression) and their proliferation/survival in 2D/3D cell assays, as a function of dox concentration and, consequently, a degree of UPS/autophagy dysfunction.

2. In a parallel approach, a set of LIR/AIM-based peptides (**Fig. 1D**) will be designed and tested for their ability to modulate autophagy in HGSC cells. LC3/AIMs are sequences harboured in a range of selective autophagy receptors, such as p62/SQSTM1 (mammalian cells) and NBR1 (both mammalian and plant cells), that bind Atg8/LC3/GABARAP proteins covalently conjugated to autophagosomes[7]. These LIR/AIMs will be synthesised as cell-permeating peptides and delivered inside the cells or, alternatively, expressed from standard or dox-inducible expression vectors. It will be assessed to which extent autophagy is activated/inhibited (depending on the design of LIR/AIMs) in HGSC cells. This approach will be performed with critical input from the partner lab of Tolga Bozkurt (ICL).
3. Methods to study autophagy in cells and tumour tissues are largely established (LC3, p62, and NBR1 staining, **Fig. 1A**). However, new techniques based on bio-sensors will be developed in collaboration with Firat Guder's lab (ICL).
4. The proteome of HGSC cells, with and without perturbed protein degradation, will be profiled using mass spectrometry (**Fig. 1E**) to define expression levels of individual proteins, and a network of proteins whose abundance changes rapidly following dox treatment will be constructed. The proteome maps and nodes will be correlated with the genomic data for the HGSC cell lines, which will be obtained using next-generation sequencing (NGS). It is expected that a significant correlation will be found between the mutational load in the genes and degradation kinetics of proteins.
5. HGSC proteins whose abundance/degradation is heavily dependent on autophagy will be studied in greater detail. It will be determined whether the mutations that destabilise them are driver or passenger ones and whether their removal of the targets by gene editing or their overexpression affects HGSC cell proliferation, etc.
6. *In vivo* experiments with the dox-inducible CRISPR/dCas9-KRAB system will be performed to test the effect of autophagy/proteasome inhibition on HGSC tumour growth in immunocompromised mice. Autophagy and cell stress biomarkers will be assessed in tumour tissues to validate the system. Manipulation of expression of the identified target proteins will also be performed to demonstrate their role in HGSC.
7. Finally, the study will be expanded onto HGSC samples obtained from patients. Autophagy and UPS status will be indirectly determined by staining tissue for ubiquitin and autophagy markers (LC3, p62/SQSTM1, NDP52, TAX1BP1, **Fig. 1A**). Several patient-derived xenograft (PDX) models will be established to test the effect of autophagy modulation on patient-derived HGSC tumour growth.

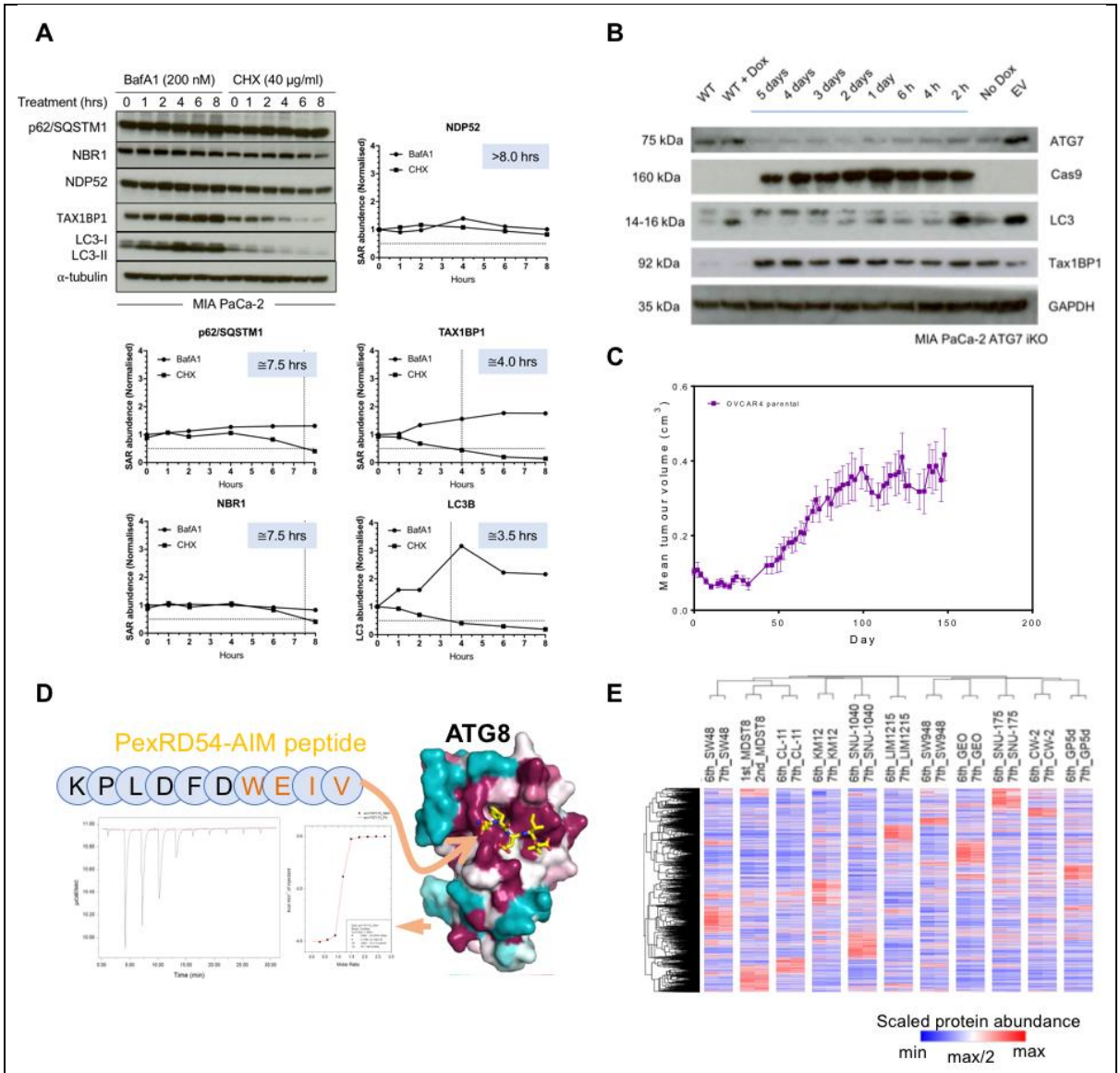

Figure 1 *Data from V. Kirkin's lab (A-C)* - **(A)** Degradation kinetics of indicated autophagy marker proteins in PDAC MIA PaCa-2 cells: TAX1BP1 as a dynamic autophagy reporter. **(B)** Analysis of the effect of *Atg7* gene silencing by the Dox-inducible dCas9-KRAB construct: autophagy inhibition is monitored by accumulation of LC3-I form and TAX1BP1 in MiaPaCa-2 cells. **(C)** **G**rowth characteristics of a HGSC cell line, OVCAR4, growing as a subcutaneous xenograft in immunocompromised NSG mice. *Data from T. Bozkurt's lab* - **(D)** PexRD54, an effector secreted by the Irish potato famine pathogen targets plant autophagy protein ATG8 through an evolutionary conserved ATG8 interacting motif (AIM/LIR): isothermal titration calorimetry (ITC) assay shows the direct one-to-one interaction between PexRD54's AIM/LIR peptide and plant ATG8; the PexRD54 AIM/LIR;ATG8 complex is also shown. *Data from J. Choudhary's lab* - **(E)** The heatmap shows the relative protein abundances profiles for 11 colon cancer (CRC) cell lines, each with a biological replicate; 10,000 proteins are identified and quantified in all the samples. The colours reflect relative abundance for each protein; it shows the reproducibility between the replicates and the protein variation between the cell lines. 20-30% of the proteome varies significantly between the different lines, these include many cancer driver genes.

Feasibility

The project designed specifically for this PhD candidate is feasible based on the multidisciplinary team of supervisors/experts available to her/him (see below for description of the individual expertise). The proposed timeline is the following:
Year 1: Making relevant constructs (some of them are already available); design of LIR/AIM peptides for autophagy modulation; making engineered HGSC cells; characterisation of engineered HGSC cells by western blot and immunofluorescence microscopy; 2D/3D assays
Year 2: Performing multiple proteomics experiments and their analysis, construction of protein networks and identification of nodes; nomination of targets; refinement of autophagy/UPS activity detection methods (collaboration with Firat Guder's lab) and use of cellular assays to prove their validity
Year 3: Making HGSC cells with changes in the target proteins identified by proteomics; testing their growth in 2D/3D assays and as xenografts *in vivo*; performing autophagy and UPS activity readouts *in vivo*
Year 4: Estabslihing and use of PDXs for validating findings obtained with HGSC cell lines; immunohistochemistry analysis of PDXs for target proteins and autophagy/UPS status; use of PDX-derived cell lines/organoids for genetic engineering and concept validation; use of bio-sensors in PDXs to validate their use in translational models.

Multidisciplinary approach

The project will utilise the joint expertise of the Institute of Cancer Research (ICR)/Imperial College London (ICL) scientists: molecular biologists (**Vladimir Kirkin ICR** and **Tolga Bozkurt ICL**), proteomics specialist (**Jyoti Choudhary ICR**), a bioengineer (**Firat Guder ICL**), and clinicians working in the area of ovarian cancer (**Susana Banerjee ICR** and **Iain McNeish ICL**).

1. **Vladimir Kirkin lab (ICR)** is experienced in autophagy (**Fig. 1A**) and ubiquitin research[8-11] and has been developing inducible dCas9-KRAB-based vector systems to inhibit autophagy (ATG7, **Fig. 1B**, ATG4B, BECN1) or the proteasome (PSMD4). They use starvation and mTOR inhibitors (TORIN, rapamycin) to experimentally activate autophagy. This lab has also developed a number of preclinical cancer models, including HGSC (which are established as both subcutaneous, **Fig. 1C,** and intraperitoneal models), that are used in the drug discovery and development cascade at the CRUK Cancer Therapeutics Unit (CTU). This lab is also experienced in cell-based assays (2D/3D) and biomarker development. Vladimir co-discovered LC3-Interacting regions/Atg8-Interacting Motifs (LIRs/AIMs)[7,12] and takes great interest in developing them further as research and therapeutic tools. The student will enroll in ICR's PhD programme and have access to the entire technical expertise of this lab. She/he will undertake all relevant ICR courses designed for the PhD students. It is anticipated that the student will spend **at least 50% of her/his time in this lab**.
2. **Tolga Bozkurt lab (ICL)** is a world-class plant research unit that has identified plant pathogen-derived LIRs/AIMs (**Fig. 1D**) that can induce or inhibit autophagy when expressed in plant cells[13,14]. The LIRs/AIMs are very well conserved in evolution[7], so that they are extremely likely to work as autophagy activators/inhibitors also in human cancer cells. In line with this view, expression of the pathogen-derived autophagy activator stimulated autophagosome formation in *C. elegans*. They will use two approaches: 1) express AIMs/LIRs from plasmids, and 2) use extracellular peptides encoding the AIM/LIR sequences to manipulate autophagy in HGSC cells. The student will spend at least **30% of her/his time in this lab**, learning about LIR/AIM biology which is conserved across the kingdoms of life. The student may choose to undertake courses offered to PhD students by the ICL.
3. **Jyoti Choudhary (ICR)** is the leader of a world-class cancer proteomics unit at the ICR. This will be instrumental for profiling the manipulated HGSC cells for their proteome changes (**Fig. 1E**). Unit's bioinformaticians will help construct protein networks and identify nodes with most significant changes during autophagy/UPS inhibition. Identified proteins will be validated in the lab of **Vladimir Kirkin** by using gain- and loss-of-function approaches. The student will learn basics of proteomics and perform the proteomics experiment under the guidance of the unit staff. In addition, basic bioinformatics training will be provided by the unit. The student will likely spend up to **20% of her/his time in this lab**.

4. **Firat Guder lab (ICL)** has developed unique bioengineering expertise that will be instrumental to develop autophagy/lysosome activity probes for monitoring autophagy/proteasomal activity in HGSC cells. The lab has developed bio-sensors that can measure immediate changes in ions/nutrients within or outside the cells. This approach may also help develop a method to score autophagy changes in *in-vivo* models. The student will benefit from the interaction with this lab and will participate in some of the lab meetings for cross-fertilization of the project.
5. **Susana Banerjee** and **Iain McNeish** will be clinical collaborators and the student's mentors in the area of ovarian cancer, which will be key for the clinical relevance of the presented PhD project. Using appropriate clinical protocols, they will help gain access to patient-derived xenografts (PDXs) that can be used to validate biomarkers and observations obtained using engineered HGSC cell lines. The student will benefit from access to the expertise of the clinical collaborators who will review her/his work and provide critical comments throughout the project.

Literature references

(Provide a bibliography of any cited literature in the proposal).

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Advertising details

(Key words or short phrases that students might type into search engines for PhD projects similar to yours).

1. Ovarian cancer
2. Autophagy
3. Proteasome

4. Proteomics
5. Bio-sensors
6. Bio-engineering

Project suitable for a student with a background in:

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- ☐ Mathematics, Statistics, Epidemiology
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