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Final Degree Project

DEVELOPMENT OF E3-INDEPENDENT PROTAC MOLECULES

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Degree in Biotechnology

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Vic, March 2020

ACKNOWLEDGMENT

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SUMMARY

Title: *Development of E3-Independent PROTAC Molecules*

Keywords: Proteolysis targeting chimera (PROTAC), 26S-Proteasome, Usp14, Ubiquitin (Ub), E3 enzyme ligase, cell clonation, VHL

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Date: June 2020

This project proposes to generate a new type of PROTAC molecules that can act independently of E3-ubiquitin ligases. The idea is to bind a target protein, which we want to degrade, directly to the proteasome. This is a major methodological difference as conventional PROTACs bind the target protein to an E3. The new PROTAC methodology we are developing avoids ubiquitination, therefore, it does not depend on the availability of E3 or ubiquitin in the cell where PROTAC acts. This project, developed in collaboration with the Research Unit on Bioactive Molecules (RUBAM), of the Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), seeks a future application in the field of pharmacology, as they can completely inactivate key proteins in multiple pathologies.

Specifically, this project has focused on the expression of the VHL target protein and the phenotypic analysis of the functionality of the USP14 26S proteasome.

RESUM

Títol: Desenvolupament de molècules PROTAC Independents d'E3
Paraules clau: Quimera dirigida a la proteólisis (PROTAC), 26S-proteasoma, USP14, ubiquitina (Ub), enzima ligasa E3, clonación celular, VHL
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Data: Juny 2020

Aquest projecte proposa generar un nou tipus de molècula PROTAC que poden actuar de manera independent de l'enzim E3 ubiquitina lligasa. La idea consisteix a unir una proteïna, que volem degradar, directament al proteosoma. Aquest fet, suposa una gran diferencia metodològica, ja que els PROTAC convencional uneixen la proteína a una E3. La nova metodologia de PROTAC que hem estat desenvolupant evita l'ubiqüitinació, i per tant no depèn de la disponibilitat d'E3 o ubiquitina a la cèl·lula on actua el PROTAC. Aquest projecte, desenvolupat en col·laboració amb la Unitat de Recerca en Molècules Bioactives (RUBAM), de l'Institut de Química Avançada de Catalunya (IQAC-CSIC), busca una futura aplicació en el camp de la farmacologia, ja que poden inactivar completament proteïnes clau en múltiples patologies. Específicament, aquest projecte s'ha centrat en l'expressió de la proteïna VHL i en l'anàlisi fenotípic de la funcionalitat del proteasoma 26S amb USP14.

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GLOSSARY

AAA+: ATPase associated with various cellular activities

ATP: Adenosine triphosphate nucleotide

BP: Base Pairs

CP: Core Particle

DUB: Deubiquitinating enzymes

EM: Electron Microscope

PROTAC: Proteolysis Targeting Chimeric

RE: Restriction Enzymes

Rpn: Regulatory Particle of non-ATPase

Rpt: Regulatory Particle of triple-ATPase

RP: Regulatory Particle

UBL: Ubiquitin-Like Protein

UBP: Ubiquitin specific protease

UPS: Ubiquitin–proteasome system

USP14: Ubiquitin carboxyl-terminal hydrolase 14

VHL: Von Hippel Lindau

WT: Wild Type

YPD: Yeast Extract Peptone Dextrose

CONTENTS

1. INTRODUCTION

1.1 THE UBIQUITIN-PROTEASOME SYSTEM

The 26S proteasome is the major protease in eukaryotic cells, responsible for protein degradation in both the cytosol and the nucleus. Ubiquitin post translational modifications target intracellular proteins, which are recognized by the proteasome. Ubiquitin modifications create a covalent bond while being attached to lysine side chains by a network of ubiquitin ligases and enzymes, and they are removed at the proteasome obtaining finally the substrate degradation. The proteasome holoenzyme is a compartmental protease of the AAA+ (ATPases associated with various cellular activities) family, and it uses ATP hydrolysis to disrupt complex structures and translocate the unfolded polypeptides into a degradation chamber for proteolytic cleavage. Due to this process eukaryotic cells can remove damaged or misfolded polypeptides. (Bedford et al., 2010)

Cellular signaling events target proteins by post translation modifications that undergo degradation.

- First proteins targeted for degradation are polyubiquitinated and recognized by proteasomal ubiquitin receptors of the 19S particle.
- Second, these proteins are unfolded and actively translocated into the proteolytic chamber via the activity of the AAA ATPase ring complex of the 19S base particle, process that requires ATP hydrolysis.
- Third, translocated proteins are degraded at the 20S by three distinct threonine-protease activities.

The ubiquitin chain acts as a signal that shuttles the target proteins to the proteasome, where the substrate is proteolytically broken down. Ubiquitylation is a reversible process, because many deubiquitylating enzymes (DUBs) are present in the cell. The ubiquitin proteasome system (UPS) controls most cellular processes such as progression through the cell cycle, signal transduction, cell death, immune responses, metabolism, protein quality control and development.

In a general view of the process, proteins targeted for degradation undergo multiple steps until free amino acids are released as final products. The first steps are ATP-dependent and performed by E1, E2, E3 enzymes and 26S proteasomes. They include ubiquitination, unfolding and translocation. The latter ATP- independent steps are: substrate deubiquitination (coupled to unfolding) and proteolytic cleavage of translocated proteins, obtaining oligopeptides. Finally oligo peptidases and exopeptidases are in charge of degrading released peptides into amino acids. Another pathway is the binding of these peptides to MHC (major histocompatibility complex) class I protects them from further degradation by exopeptidases. (Nandi et al., 2006) (Figure 1)

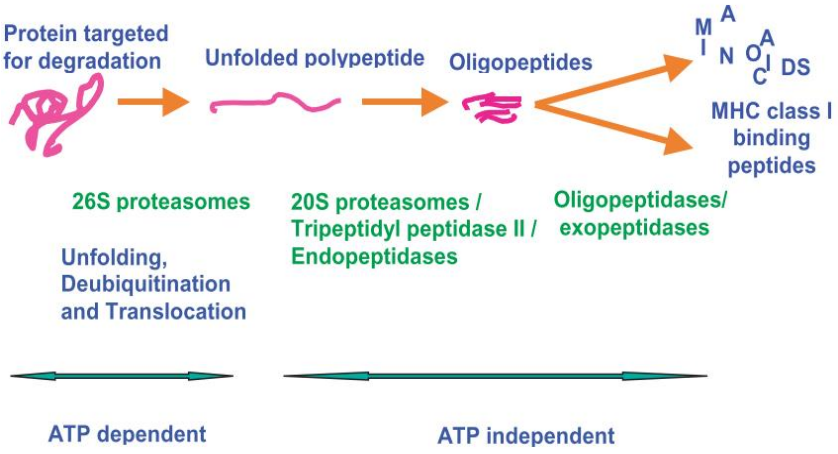


Figure 1; Protein degradation. The image has been extracted from *"The ubiquitin-proteasome system",* of Dipankar Nandi, Pankaj Tahiliani, Anujith Kumar, and Dilip Chandu, 2006 NCBI. Copyrigth.

1.1.1 The Ubiquitination System

ATP dependent 26 S proteasomes require proteins targeted with ubiquitin. Ubiquitin is a protein composed of 76 amino acids. To get the covalent bound between ubiquitin and the protein, an isopeptide linkage between the carboxy terminal glycine of ubiquitin and, usually, the ε-amino group of lysine in the target protein must take place. To form a polyubiquitin chain, similar isopeptide linkage is formed between the carboxy terminus of ubiquitin with the ε-amino group of lysine of another ubiquitin molecule.

The ubiquitin process is followed by these steps: an activating enzyme, E1, transfers ubiquitin to a carrier E2 enzyme, which in turn tags ubiquitin to the substrate with the help of E3 enzymes. E3 are ubiquitin ligases enzymes, which specifically recognize protein targets, leading to their ubiquitination and subsequent degradation. It must be taken into account that further polyubiquitination is required to target proteins for degradation.

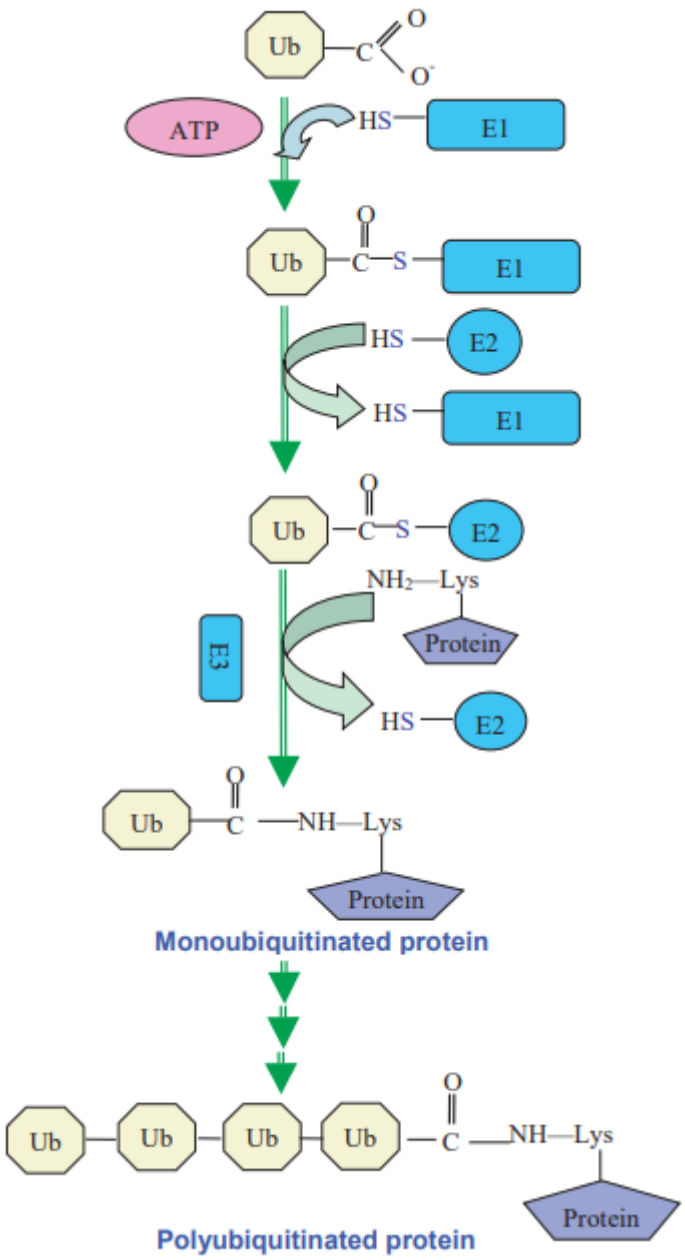


Figure 2. Peptide Ubiquitination Process. The image has been extracted from *"The ubiquitin-proteasome system",* of Dipankar Nandi, Pankaj Tahiliani, Anujith Kumar, and Dilip Chandu, 2006 NCBI. Copyrigth.

To get this process further developed (Figure 2), first as explained we need the activation of ubiquitin resulting in (ubiquitin-AMP as an intermediate) ubiquitin-E1 thiol as a product, this activation is carried out by E1 enzyme and it involves the presence of ATP. E2 are capable of recognizing E1 ubiquitin thiol ester and ubiquitin is transferred by another thiol ester bond. The function of E2, also called as ubiquitin-carrier proteins, is to aid in the carrying of the previously activated ubiquitin to the substrate. E2 enzymes associate with E3 enzymes, which are responsible for the final target selection and specificity. It has been proved that the ratio of genes encoding the ligases are hundreds of E3, tens E2 and few genes of E1. Once the process of ubiquitination modified proteins may be signalized to distinct pathways, depending on the final modification, monoubiquitination or polyubiquitination, and on the internal lysine involved in the polyubiquitin chain formed. The most common modification in eukaryote cell is lysine-48 polyubiquitnation, which signalizes proteins to the proteasome for degradation. (Nandi et al., 2006)

1.2 THE PROTEASOME

As previously seen, proteasome is a large protein complex that requires metabolic energy for the degradation of intracellular proteins. Polymerization of ubiquitin serves as a degradation signal for several target proteins. (Figure 3)

The proteolytic activity of the proteasome is essential to regulate multiple cellular processes, including cell cycle, DNA replication, transcription, signal transduction, and stress response. (Tanaka, 2009)

Proteasome structure can be divided into the 20S Core Particle (CP) and the 19S Regulatory Particle (RP), which is composed of the base and lid subcomplexes. (Bedford et al., 2010)

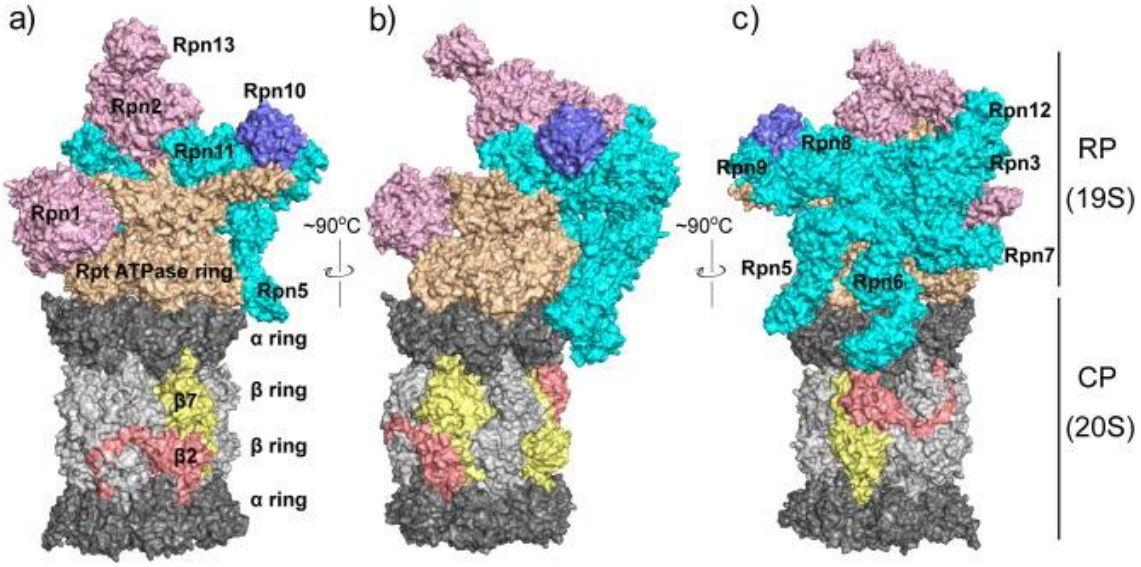


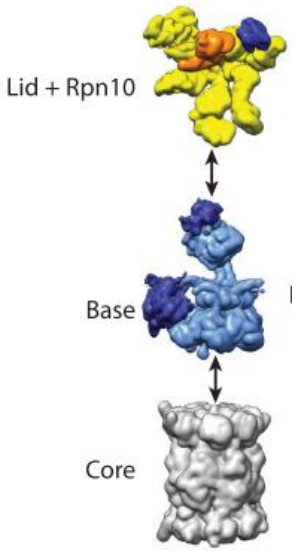
Figure 3; The structure of the 26S proteasome. Grey corresponds to the CP . The Rpt ATPase ring subunits of the RP base are colored light brown, while the Rpn1, Rpn2 and Rpn13 base subunits are pink. Rpn10 is blue. The lid subunits of the RP (19S) are colored in light blue. A, B and C images, show different angles of the proteasome. The image has been extracted from "*Proteasome Structure and Assembly*", Lauren Budenholzer, Chin Leng Cheng, Yanjie Li, and Mark Hochstrasser, 2017, NCBI. Copyrigth.

Subcomplex	*Saccharomyces cerevisiae*	*Homo sapiens*	Function
BASE	Rpn1	PSMD2/S1	Ubp6 and ubiquitin / UBL binding
	Rpn2	PSMD1/S1	Structural
	Rpn13	ADRM1	Ubiquitin / UBL binding
	Rpt1	PSMC2/S7	ATPase
	Rpt2	PSMC1/S1	ATPase
	Rpt3	PSMC4/S6	ATPase
	Rpt4	PSMC6/S10	ATPase
	Rpt5	PSMC3/S6a	ATPase
	Rpt6	PSMC5/S8	ATPase
LID	Rpn3	PSMD3/S3	Structural
	Rpn5	PSMD12	Structural
	Rpn6	PSMD11/S9	Structural
	Rpn7	PSMD6/S10	Structural
	Rpn8	PSMD7/S12	Structural
	Rpn9	PSMD13/S11	Structural
	Rpn11	PSMD14/Poh1/Pad1	Deubiquitinase
	Rpn12	PSMD8/S14	Structural
	Sem1	PSMD9/Dss/Rpn15	Structural
ADDITIONAL COFACTOR	Rpn10	PSMD4/S5a	Ubiquitin / UBL binding
ASSOCIATED DEUBIQUITINASES	Ubp6	USP14	Deubiquitinase
	NA	Uch37	Deubiquitinase

Table 1; 26S subcomplexes and its functions. The table has been extracted from "*Structure and Function of the 26S Proteasome*", of Jared A.M. Bard, Ellen A. Goodall, Eric R. Greene, Erik Jonsson, Ken C. Dong, 2010, NCBI. Copyright

1.2.1 Structure of the proteasome

1.2.1.1 Core Particle (20S Proteasome)



The CP is a cylinder protein complex with a molecular mass of approximately 750 kDa. X-Ray crystallography studies have shown that this complex form a packed particle, as a result of axial stacking of two outer α rings and two inner β rings, which are made up of seven structurally similar subunits. Moreover the center of the α ring is almost closed, preventing proteins from having access into the inner chamber of the β ring that contains the proteolytically active sites. This mechanism allow substrate to have access to the active sites only when the center of α rings are opened. . The mechanisms that regulate the opening of α rings are poorly known, but binding denature proteins seems to help the process. The 20S proteasome degrades target proteins, generating oligopeptides. The peptide products are hydrolyzed to amino acids by the enzymes oligopeptidases and amino-carboxyl peptidases. (Tanaka, 2009)

Figure 4; Lid, Base and Core. This image has been extracted from "*Structure and Function of the 26S Proteasome*" Jared A.M. Bard,Ellen A. Goodall, Eric R. Greene,Erik Jonsson, Ken C. Dong, and Andreas Martin, Annual Reviews, 2010. Copyright.

1.2.1.2 Regulatory Particle (19 S Proteasome)

The proteasome is capped on the ends of the central 20S proteasomal core by regulatory proteins. The RP is differenced into two subcomplexes which are the lid and the base, in turn, these are made up of both Rpt and Rpn subunits, ergo ATP and non ATP domains.

RP recognizes polyubiquitin-marked proteins, removes the chain and entraps the protein moiety, unfolds the substrate proteins, and finally opens the α-ring to transfers the unfolded substrates into the CP for its posterior degradation. (Tanaka, 2009)

1.2.1.2.1 Lid

The lid subcomplex acts as a platform that braces one side of the base. The main function of the lid is to deubiquitylate the captured substrates. The lid includes subunits that have structural function (Rpn3, Rpn5, Rpn6, Rpn7, Rpn9, Rpn12, and Rpn8) and a subunit Rpn11 that is a DUB, deubiquitinating enzyme. Rpn11 is responsible for the removal of substrate-attached ubiquitin chains

before they enter the AAA+ ATPase. Rpn11 DUB cleaves the polyubiquitin chain at a proximal site, which is then cleaved into monomeric ubiquitin by other DUBs.

There is present in the proteasome as well a DUB which is Ubiquitin carboxyl-terminal hydrolase 6 (Ubp6). Ubp6, Ubiquitin carboxyl-terminal hydrolase 14 (USP14) in mammals, is an ubiquitin-specific protease that binds to Rpn1 (subunit of the base) and uses an active site cysteine to cleave ubiquitin chains from substrates. Ubp6 is induced by ubiquitin deficiency. (Bedford et al., 2010)

1.2.1.2.2 Base

The base complex of proteasomes has three functional roles: capturing target proteins via ubiquitin recognition, promoting substrate unfolding and opening the channel in the α ring.

The base of the proteasome includes three non-ATPase subunits (Rpn1, Rpn2, and Rpn13). Rpn1 contain large α solenoids with different binding sites for ubiquitin proteins (UBLs), Rpn2 contains them as well and a binding site for the ubiquitin receptor Rpn13.

Rpn10 is a UBL receptor shuttle particle that transiently binds to the proteasome. Moreover it acts as a bridge between subcomplexes. In addition, at the center of the base, the proteasome contains six ATPase subunits (Rpt1–Rpt6), which form the ring-shaped heterohexameric motor of the proteasome, it facilitates the opening of the gate allowing substrate to reach the catalytic sites. The method followed is based on the Rpts that use its AAA+ domains at the central pore of the motor to engage proteins substrates and unfold them, then translocate the proteins into the 20S core. Substrate engagement is accompanied by a major conformational change that switches the RP into a state ideally suited for processive protein translocation. (Tanaka, 2009)

1.2.2 Mechanism: conformations linked with phases:

1.2.2.1 Ubiquitin recognition, deubiquitination and unfolding:

It has been studied and shown by EM structural data that proteasomal peptide hydrolysis, ATP hydrolysis, and deubiquitination activities, maintain an intimate relationship.

As explained before, substrate engagement involves a major conformational change that switches the RP into a state ideally suited for substrate translocation.

Initially, two conformations were identified in the proteasome: the s1, substrate-free state and the s3 the substrate-processing state. Recent studies show up to seven conformations of proteasome states. In yeast the main proteasomal conformations are s1, s2, s3, s4 and in human are closely related termed as SA,SB,SC,and SD.

S1 conformation is also likely the resting state of the proteasome, as it is the major conformation observed for proteasomes in the absence of added substrate. The other conformations are induced once the proteasome has to perform a substrate degradation or in the presence of ATP analogs.
Through the four conformations the core remains unchanged, but the orientation of the base and lit between them and with the core change extremely.

- Ubiquitin receptors: Rpn10, Rpn13 Rpn1

Intrinsic or extrinsic ubiquitin receptors are needed to recruit the substrate that the proteasome requires for its posterior engagement with the AAA+motor, taking advantage of the ubiquitin modifications. Firstly, intrinsic receptors of the proteasome are Rpn10, Rpn13 and Rpn1, which have some flexibility and allow the proteasome to recognize different substrates with several geometries of ubiquitin chains. On the second place, in addition to the ubiquitin receptors recently mentioned, there is the presence of several extrinsic receptors, which are able of deliver substrates through dynamic interactions with both the proteasome and ubiquitin chains. Extrinsic receptors allow the proteasome to accommodate an even wider variety of substrate geometries and ubiquitin modifications than just with intrinsic receptors.
Relating these receptors with the different conformations, it has been observed that between s1 and s3, Rpn1 receptor changes its interactions Rpt1 and Rpt2, as a consequence it rotates relative to the ATPase ring and the central pore. This rotation allows Ubp6 to make stable contacts with the base ATPase site, stimulating Ubp6 DUB's activity in non-s1 states. During the same transitions, Rpn10 makes additional contacts with Rpt4 and Rpt5.
The functional importance of these ubiquitin-receptor transitions has yet to be determined, but it is assured that the different positions may influence the proteasome's affinity for substrates to enter to the central pore.

- DUBs: Process; Rpn11, (Ubp6:USP14))

The first step is performed as previously explained by ubiquitin receptors, which are able of recognizing and capturing substrates marked with ubiquitin. For this reason, proteasome contains as well DUBs, which are capable of editing and removing the ubiquitin signal.

- Rpn11

Rpn11 is the integral proteasomal DUB. Its function is essential for proteasome activity and cell viability. Rpn11 contains a catalytic zinc ion and it resides just above the N-ring of the AAA+ motor and it is adjacent to the ubiquitin receptor Rpn10. The Rpn11 deubiquitinase function is active after the substrate is engaged, but before the AAA+ ATPase would unfold the protein. The mechanism of

action of Rpn11 is by removing ubiquitin modifications in block by hydrolyzing the isopeptide bond at the base of the chain between the substrate lysine and the C terminus of the first ubiquitin moiety. During the transition from s1 to s2, there lid rotates and Rpn11 DUB moves from an offset to a position above the central processing pore of the base. This position might seems ideal for translocation-coupled deubiquitination, but it restricts substrate access to the central pore, leading to propose that only the s1 state is capable of efficiently engaging a polypeptide with its translocation machinery, therefore, s1 represents the primary substrate-binding conformation.

- Ubp6:USP14

Ubp6, known as USP14 in humans is a DUB, considered to be the allosteric regulator of proteasome. Ubp6 function is to act as a deubiquitinase, but it has been studied that by deleting Ubp6 from the proteasome, the degradation of model substrates in vitro was accelerated and it was not lethal for the specie (*S. cerevisiae*). Several limitations in vivo were found like showing a defect in growth owing to increased degradation of substrates.
Concurrently, the bound between Ubp6 and ubiquitinated protein stimulates the ATPase activity and 20S gate opening and inhibits substrate engagement by destabilizing the s1 state.
Nevertheless, Ubp6 cleaves long chains better than does Rpn11, and it can cleave ubiquitin chains only when more than one chain is attached to a substrate. Even though it has a highly and strong deubiquitinase activity, it cannot substitute for Rpn11.

- Unfolding: ATPase ring

Differences between conformations, remain, among others, in the arrangement of the AAA+ domains. Substrate engagement induces the transition from s1 to s3 or s4, where the contacts between AAA+ subdomains are fully formed, therefore, substrate engagement increases the ATPase activity of the Rpt motor.
In s1 conformation, the base of the proteasome hydrolyzes ATP slowly, but in s2 and s3 the high concentrations of ADP effects on proteasome's conformation.

Unfolding and translocation of protein substrates into the proteolytic core is driven by the Rpt1- Rpt6 heterohexameric motor. The base of this mechanism is to convert chemical energy of ATP hydrolysis into mechanical work. Domains of Rpt1–Rpt6 form the N-ring, which is essential for the structural stability of the hexamer. The AAA+ motor pulls the protein substrates during mechanical threading to induce their unfolding. When ATP hydrolysis is performed, the AAA+ hexamer change its typical conformation of six rigid bodies that are connected by linkers between the large and small subdomains.

Rpt ring is highly dynamic, its structure varies differentially above all between the s1 and s3 states. In the s1 state, rigid-body interactions between Rpt subunits are absent. For the other conformations, the ring of ATPases flattens out and adopts a shallower staircase arrangement.
(Bedford et al., 2010)

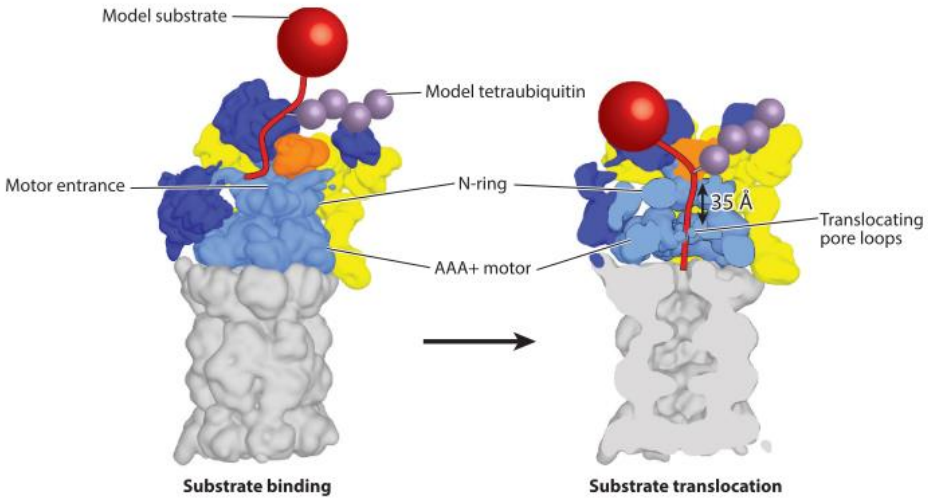


Figure 5; Model for substrate engagement by the proteasome. In the left image proteasome is shown in the s1 state with a model substrate (red) tethered through a tetraubiquitin chain (purple). In the right one Substrate engagement shifts the proteasome to the s3 or s4 state. This image has been extracted from "*Structure and Function of the 26S Proteasome*" Jared A.M. Bard,Ellen A. Goodall, Eric R. Greene,Erik Jonsson, Ken C. Dong, and Andreas Martin, Annual Reviews, 2010. Copyright.

1.3 PROTACs

PROTAC, proteolysis targeting chimera is an artificial molecule that utilizes the proteasome-mediated proteolysis, using the E3 ligase function for ubiquitination to selectively degrade the pathogenic proteins. PROTAC is a heterobifunctional molecule that consists of a linker conjugating both the target protein and the E3 ligase, obtaining the ubiquitination of a concrete protein, which will be posterior degraded by the proteasome. The linker connects two heads a small peptide or molecule for E3 ubiquitin ligase recognition, and a ligand for target protein recognition. (*PROTAC Molecule Discovery - Creative Biolabs*, 2020).

PROTACs can degrade the target pathogenic proteins and regulate its signaling pathways. Those are employed in the degradation of different types of target proteins related to various diseases, including cancer, viral infection, immune disorders, and neurodegenerative diseases.(Gao et al., 2020).

This type of molecule is unique conventional inhibitors need an almost perfect degree of target engagement during an extended period of time, whereas PROTACs can degrade molecules and it will

have fast rates of degradation and relatively short exposure. (*What Are PROTACs and How Do They Treat Diseases? | The New York Academy of Sciences*, 2019).

1.3.1 E3 Based PROTACs

PROTACs are heterobifunctional small molecules based on three chemical elements: a ligand binding to a target protein, a ligand binding to E3 ubiquitin ligase, and a linker for conjugating these two ligands. Those are responsible of degradation of the target protein through the ubiquitin-proteasome system. (Gao et al., 2020) (Figure 3)

PROTACs present an improve method for proteolysis since they have the potential to eradicate "undruggable" protein targets, like transcription factors and non-enzymatic proteins, which are not limited to physiological substrates of the ubiquitin-proteasome system.

Even so, this technology presented several limitations, involved with poor stability, biodistribution, and undesirable off-target effects. Another limitation is related with the choice of the most effective ligase for a particular peptide, which is a notable limitation since human genome encodes more than 600 E3 ubiquitin ligases.(Gu et al., 2018)

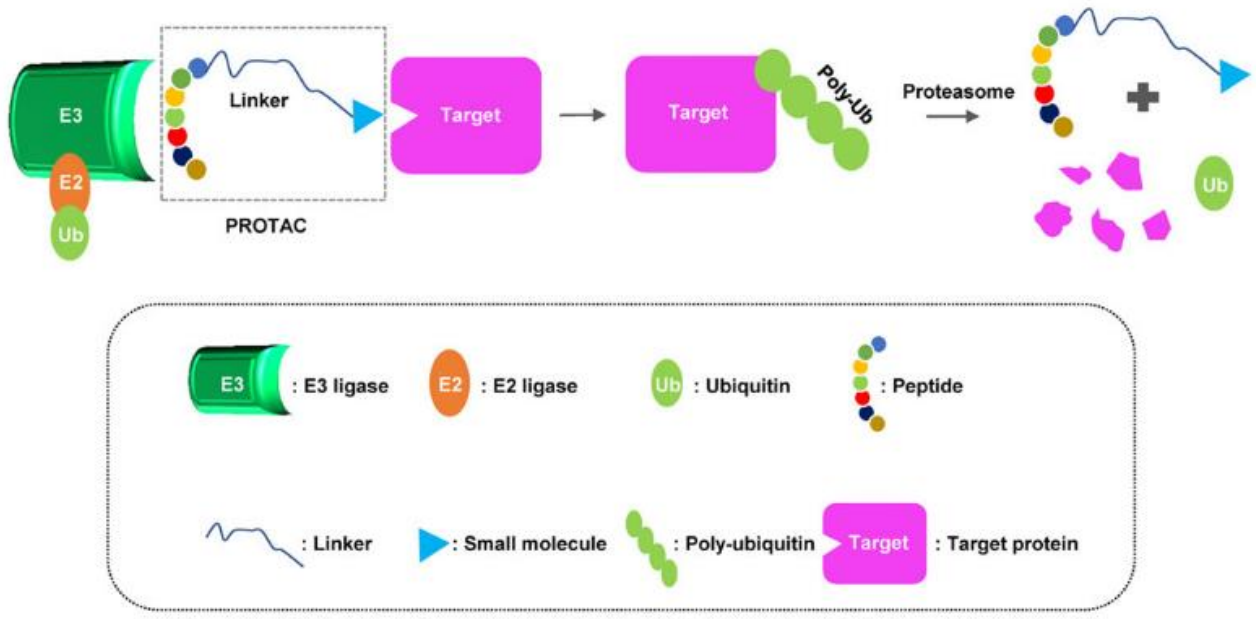


Figure 6; PROTAC's representation. The image has been extracted from "*The PROTAC technology in drug development*", Yutian Zou, Danhui Ma1, Yinyin Wang, 2019, NCBI. Copyright.

1.3.2 E3-Independent Protacs

New PROTACs have been developed since the E3 based PROTACS, known as E3-Independent PROTACS, meaning that the use of the molecule is not to be marked with the ubiquitin thanks to the activity of the E3 ligase, and further degraded by the proteasome, but to directly using the catalytic activity of the proteasome skipping the ubiquitination process. The result of this new type of PROTACS are more direct peptide degradation and a more effective process.
In this project we describe an E3-Independent PROTAC-like molecule as a new method that allows to bypass the ubiquitination step.
Taking into account that USP14 is a proteasome DUB and IU1 is its chemical inhibitor, which acts as a proteasome activator. It has been proved that DUBs has a conserved structure and this leads researchers to look for its covalent inhibitor. This method proposes IU1 to bind USP14, being used as a hole proteasome binder. On the other side of the linker the target protein which will be removed in the future, is attached. We propose to degrade the Von Hippel Lindau (VHL) ubiquitin, which has been previously validated in PROTAC studies.
The aim of this project is to create a USP14 binding to the hole proteasome – VHL interactor, to trigger its degradation.
Gascon, M. (2019). *Development of a benchmark for testing E3-Independent PROTAC-like molecules* (Unpublished Master's Thesis). Pompeu Fabra University, Catalunya.

The new PROTAC methodology we are developing avoids ubiquitination dependence, therefore, it does not depend on the availability of E3 or ubiquitin in the cell where PROTAC acts. Direct signaling to the proteasome should allow us to bypass the ubiquitination step thus simplifying the system, improving kinetics, and reducing off-target effects.
A future application in the pharmacological field is sought, since key proteins can be completely inactivated in multiple pathologies.

1.4 PREVIOUS RESEARCH

As explained above, the objective of this new PROTAC is to use the previous E3 binding site, as IU1, that is, as an inhibitor of USP14, meaning that PROTAC could be attached to the entire proteasome. The other side of the linker will be bind to VHL protein.

1.4.1 Ubp6:USP14

IU1 has been used for the inhibition of Ubiquitin Specific Peptidase 14 (USP14) in human cells. For this reason the first aim of the project was to substitute Ubp6 for USP14 which is the homologous protein in humans, since the model we are working with is *Saccharomyces cerevisiae*. The creation

of a humanized proteasome was achieved by depletion of Ubp6 gene and posterior plasmid insertion of its homolog USP14 which was preceded by Rpn10 promoter. (Zuin et al., 2015).

Cell sensitivity to cycloheximide was also performed to ensure that this process could be recreated *in vivo* and to have the evidence if USP14 could have the same function as Ubp6 in the yeast system. The result proved that Ubp6D strains that expressed USP14 recovered equally well than the wild type ones, leading us to consider that humanized proteasome was able to at least partially function as a wild-type proteasome.

The process followed was to deplete Ubp6 gene from the strains to avoid homolog recombination, clone USP14 gene under Rpn10 promoter in order to achieve its expression and thirdly, check complementarity between both DUB's *in vivo* aiming to know if USP14 was able to recover Ubp6 function when the latter was depleted.

Gascon, M. (2019). *Development of a benchmark for testing E3-Independent PROTAC-like molecules (*Unpublished Master's Thesis). Pompeu Fabra University, Catalunya.

1.4.2 VHL

The VHL protein likely plays a role in other cellular functions, including the regulation of other genes and control of cell division. It is classified as a tumor suppressor gene.

Since several PROTACS have been developed against VHL, we considered these suitable to constitute the effector moiety of our bifunctional PROTAC.

Parallel to the new proteasome composition, an attempt was made to express the VHL protein *in vivo*, with a GAL promoter, under galactose induction. No results were obtained that expressed that our buddy yeast cells expressed the VHL protein, for this reason it was decided to change the promoter.

2. OBJECTIVES

The main objective of this project is to finally reach an E3-Independent PROTAC functional molecule in order to optimize and improve the protein degradation process.

To get this main goal, several partial objectives must be taken into account:

- Expression *in vivo* of the target protein VHL (Von Hippel Lindau) to be further degraded.
- Test which cloning system is more effective whereas if it is normal cloning based on digestion with particular restriction enzymes, ligation and transformation or Gap repair strategy, based on homologous recombination.
- Test Ubp6:USP14 homology in PROTACs' by validation mediated by fluorescence assays.

Secondary objectives of this project are to deeply understand the mechanisms of proteasome and peptide degradation and to get familiarized with the function of each subcomplex and proteasome domain.

In third place, expand my knowledge of bioinformatics in order to create 3-dimensional images with bioinformatic tools, which might increase the clarity of proteasome and of target protein VHL structure.

3. MATERIALS AND METHODS

3.1 PLASMID PRESENTATION

This project has focused on the cellular cloning of the VHL protein under the Prom Rpn10. For the realization of the study different vectors were used, below there is presented the structure of those two with which we have obtained some final results.

BC417: pESC GFP-VHL

This plasmid present the VHL gene, which is intended to clonate under Rpn10 promotor.
This image shows the genes that are included in this vector as well as the restriction sites that it contains.

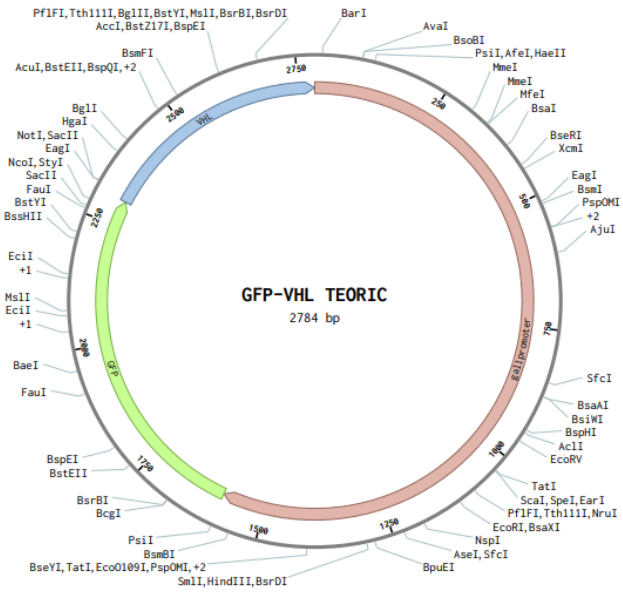


Figure 7; *Construct pESC GFP-VHL, in Benchling. Own source.*

BCO406: pRS425 PromRpn10 USP14; LEU Auxotrophy
BCO404: pRS424 PromRpn10 USP14; TRP Auxotrophy

The following plasmids will constitute the structure that will be finally cloned. The intention is to eliminate the USP14 gene in the two plasmids. The main difference between these two lies in the auxotrophy, in one case it is Leucine (LEU) and in the other Uracil (URA).

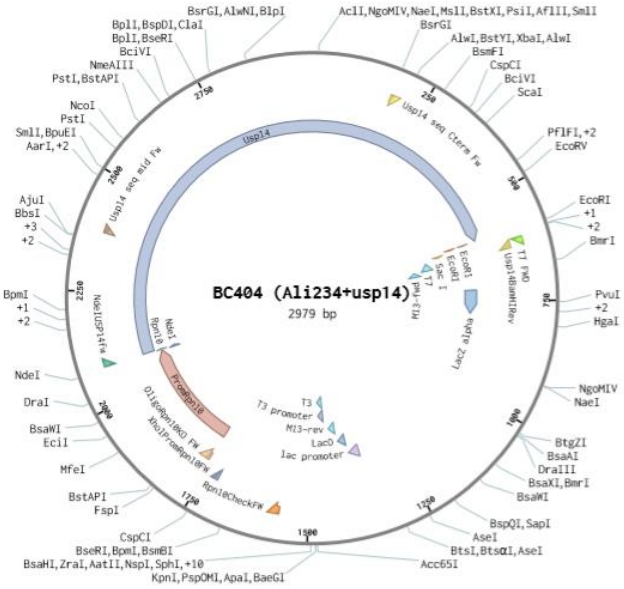


Figure 8; Construct pRS424 PromRpn10 USP14; TRP Auxotrophy, in Benchling Own source.

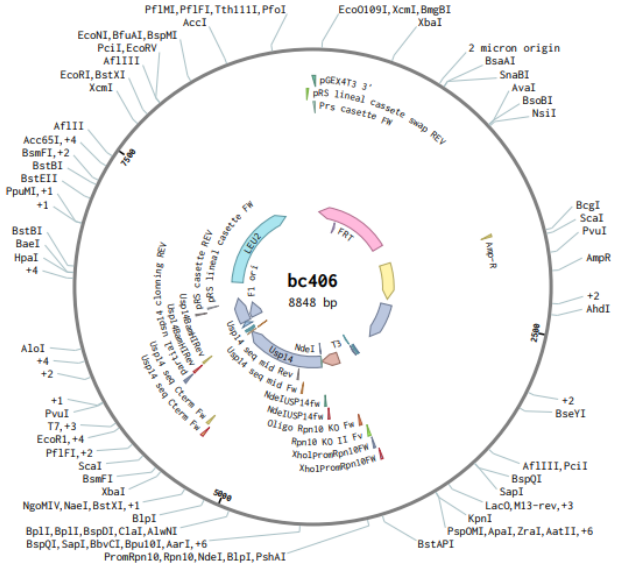


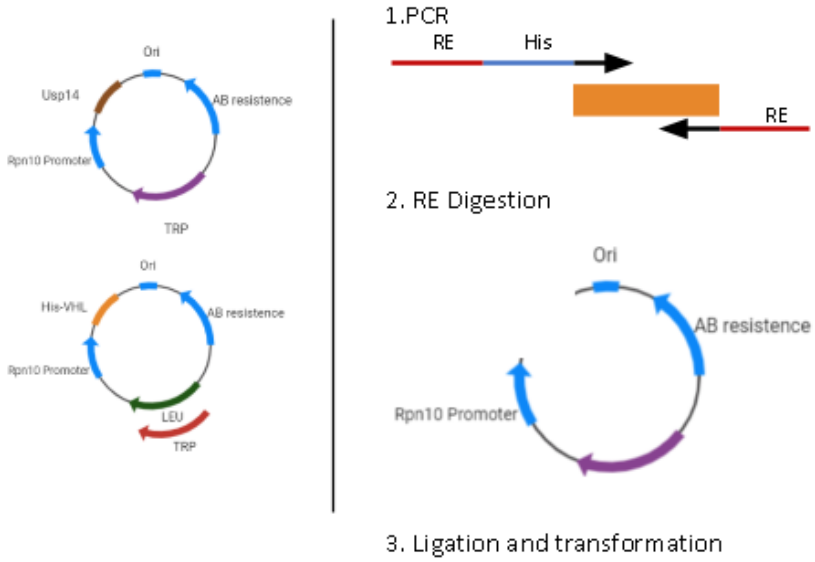
Figure 9; Construct pRS425 PromRpn10 USP14; LEU Auxotrophy, in Benchling Own source.

3.2 CLONATION METHODS

In this section we will present the different steps that have been followed and the different methodologies used to finally obtain cloning. Differentiating between the steps of strategy of basic clonation or gap repair.

3.2.1 Basic Clonation

First, it was implemented the basic cloning strategy.



The follow image shows graphically which steps have been followed and in what order.

Figure 10; Basic clonation strategy His-VHL. Own source.

3.2.1.1 PCR AMPLIFICATION

In order to amplify the His-VHL region, a PCR technique is used, by this technique a large number of copies of a particular DNA fragment are obtained. High amount of copies of VHL gene were needed for the clonation. The following primers were used:

Primer	***Sequence***	***Fwd-Rev***	***Description***
518	*GGAATTCCATATGCATCACCATC ACCATCACATGGAGGCCGGGCG GCCGCG*	FW	NDEI-HIS6-VHL
520	*CGCGGATCCCTAATCTCCCATC CGTTGAT*	Rev	VHL-STOP-BAMHI-SPACER

Table 2; Primers for PCR Amplification

The primers result in fragments of about 529 bp of the VHL + 6His + Gene regions suitable for the restriction enzymes used for subsequent digestion. The recommended temperature to amplify these primes is 42ºC.

A final volume of 50 µL was used to perform PCR amplification.

Reagents	**Final Volume of 50 µL**
Buffer 10 X	5 µL
MgSO4	3 µL
dNTP	5 µL
H2O	36,5 µL
RW (1:20)	1,5 µL
REV (1:20)	1,5 µL
DNA sample	0,5 µL
KOD enzyme	1 µL

Table 3; PCR reagents and volumes

The temperatures and time for the gene was optimized until the ones that gave the best results were found. To check the results of the PCR and see the length of the DNA fragments, an electrophoresis is performed with a 1% agarose gel.

3.2.1.2 DIGESTION

Next, what is intended is to digest the vector that contains the Rpn10 promoter, eliminating USP14, which will subsequently be replaced by the VHL gene with the histidine tail for detection.
First of all, it was decided which restriction sites will be the most suitable, to digest USP14, taking into account which RE we have available. In order to check if the digestions are correct individually, we also do partial digestions.

Reagents	**Total Digestion (50 µL)**	**Partial Digestion (25 µL)**	**Partial Digestion (25 µL)**
DNA	2 µL	1 µL	1 µL
Buffer 10 X	5 µL	2,5 µL	2,5 µL
RE (NdeI)	1 µL	0,5 µL	
RE (BAMHI)	1 µL		0,5 µL
H2O	41 µL	21 µL	21 µL

Table 4; Reagents and volumes for total and parcials Digestions

Finally, we incubate at 37 ºC 1h: 30 and 1 µL of alkaline phosphatase, to the digestion of the vector and incubate at 37 ºC for another 15 minutes.

3.2.1.3 LIGATION

Then, the fragments obtained from the pcr and the digested plasmid, must be joined to form the resulting plasmid that is being tried to obtain. To achieve it, the ligation was performed from the T4 Ligase enzyme.

In first place set up the following reaction in a microcentrifuge tube on ice:

Reagents	**20 µL Final Volume Reaction**
T4 DNA Ligase Buffer (10X)	2 µL
Vector DNA (4 kb)	50 ng
Insert DNA (1 kb)	37,5 ng
MiliQ H2O	To 20 µL
T4 DNA Ligase Enzyme	1 µL

Table 5; Reagents and volumes for Ligation

Gently mix the reaction by pipetting up and down and microfuge briefly and incubate in room temperature for 10 minutes.

3.2.1.4 TRANSFORMATION

Transformation is a process of horizontal gene transfer by which cells take up foreign genetic material from the environment. It is the ability to take up free, extracellular genetic material. Transformation consists of the genetic alteration of a cell resulting from incorporation of exogenous genetic material through the cell membrane. (*Bacterial Transformation | Sigma-Aldrich*, 2020)

3.2.1.4.1 Yeast Transformation

All this process must be performed under sterile conditions. This process is based in the lithium acetate method.

In first place we have to inoculate 3-5 mL of Yeast Extract Peptone Dextrose (YPD) medium with a single colony and incubate it at 30 ºC overnight. Measure the OD600 which must be around 1. Transfer 50 mL to a falcon 50 and centrifuge at 1300 rpm 3'. Discard the supernatant. Add 1 Ml of sterile water to re-suspend the cells and finally transfer the suspension to an Eppendorf. Later centrifuge at 2000 rpm 1', then repeat the step and wash it. Meanwhile boil salmon sperm for 5'. Add 1 Ml of TEL and suspend the cells. Centrifuge again at 2000 rpm for 1 minute, to pour off the TEL. Add 500 µL of TEL and suspend the cells. Take 50 µL of cells for transformation and transfer it to a new Eppendorf. Add 5 µL of previously boiled sperm and then add 1 µg of the plasmid. Then add 350 µL of TEL-PEG and mix well the transformation mix (closing previously the Eppendorf with Parafilm). Remove TEL-PEG and re-suspend cells in 1 Ml of YPD. Incubate for 3-4 ' at 30 ºC. Centrifuge again at 2000 rpm for 1' and remove completely the YPD. Add 50 µL of sterile H2O and re-suspend the cells. Finally plate them on YPD+ KAN Antibiotic plates.

3.2.1.4.2 Bacteria Transformation

After transformation into yeast, in order to amplify our vector, it was proceed to transform into bacteria, for this it was used the protocol for high efficiency transformation for NEB Stable Competent E.coli.

In first place thaw a tube of competent E. coli cells on ice for 10 minutes. In second place add 2 µL of containing DNA plasmid to the cell mixture and mix it 4-5 times, without vortex. Place the mixture on ice for 30 minutes and rapidly perform the heat shock at 42°C for 30 seconds. Then place on ice for 5 minutes. Add 950 µl of room temperature media to the tube and place it at 37°C for 60 minutes in shaking conditions. Finally spread 50–100 µL of the cells and ligation mixture onto the plates. Finally incubate it overnight at 37°C.

3.2.1.5 MINIPREP

After getting the transformation performed, in both strategies, it is used the MiniPrep kit to obtain the DNA from the transformed cells. We differentiate between yeast MP and MP bacteria, depending on which cell line we are working on.

3.2.1.5.1 Bacterial MiniPrep

The kit we are using is NZY Miniprep.

1. Cultivate Bacterial cells: Pellet 1-5 mL of E.Coli and discard supernatant, removing as much media as possible.
2. Cell lysis: by re-suspending cell pellet in 250 µL Buffer A1 and transfer it to an Eppendorf. Add 250 µL of Buffer A2 and mix gently and incubate it at temperature room for 4 minutes. Later add 300 µL of Buffer A3 and mix again, by inverting.
3. Clarification of lysate: Centrifuge for 5-10 min at room temperature
4. Bind DNA: Place NZYTech spin column in 2 mL collecting tube, and load the supernatant obtained, centrifuge it for 1 min at 11.000 xg. Discarding the flow-through.
5. Wash silica membrane: Add 500 µL of Buffer AY into the column and centrifuge for 1 min (discarding the flow-through). Following add 600 µL of Buffer A4 (with ethanol previously added), centrifuge and discard flow-through.
6. Dry silica membrane: Re-insert the NZYTech spin column into the empty 2 Ml collecting tube and centrifuge for 2 min.
7: Elute highly pure DNA: Place the dried NZYTech spin column into a clear 1.5 mL Eppendorf and elute in 50 µL of Buffer AE (in some experiment we have eluted at 30 µL in order to concentrate the sample more). Incubate it for 1 min at room temperature and centrifuge for 1 min.

3.2.1.5.2 Yeast Miniprep

In order to perform Yeast MiniPrep, first we have to take into account that is less effective than the previous one. In first place, inoculate a single colony into 5 Ml YPD in a glass tube. Grow them overnight at 250 rpm, 30 ºC. Centrifuge 3 mL of the culture for 30s at top speed. Re-suspend in 300 µL of resuspension buffer add 100 µL of glass beads. Vortex at maximum speed for 5 min and boil at 95 ºC for 3 min. Then vortex 2 min and boil again 3 min. Place on ice 1 min and spin 5 min at 20.000 g. Transfer the supernatant to a new Eppendorf and add the lysis buffer and continue with the bacterial MP protocol.

After MiniPrep Kit is used and DNA is eluted and concentrated, it is necessary to look at the concentration of the elute. This step is done in Thermo Scientific™ NanoDrop™. NanoDrop microvolume system describe an effective nucleic acid quantification method.

3.2.1 Homologous Recombination Clonation

This clonation technique is based on homologous recombination which consists on the exchange of genetic material between two strands of DNA that contain long stretches of similar base sequences. Foreign DNA flanked by sequences identical to the ones upstream and downstream of the target gene's location is introduced into a cell. The cell recognizes the identical flanking sequences as homologues, causing target gene DNA to be swapped with the foreign DNA sequence during replication. (Homologous Recombination | Biology | Britannica, 2020).

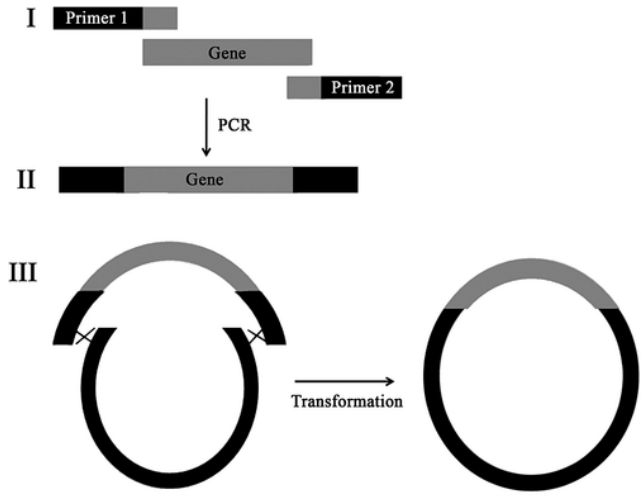


Figure 11; Gap repair method transformation. The image has been extracted from *"Improved gap repair cloning in yeast: treatment of the gapped vector with Taq DNA polymerase avoids vector self-ligation*", Daniela Bessa,, Filipa Pereira, Roxana Moreira, Björn Johansson and Odília Queirós, 2012, NCBI. Copyright.

The methodology followed for the gap repair cloning technique in yeast, recently explained, is very similar to the one used for the basic cloning strategy. The first two steps, PCR and vector digestion, are the same, but including the amplification of a homologous sequence, which will be the key for homologous recombination. Ligation step individually is not required, since it is performed along transformation thanks to the guide of homologous sequences. (Figure 12)

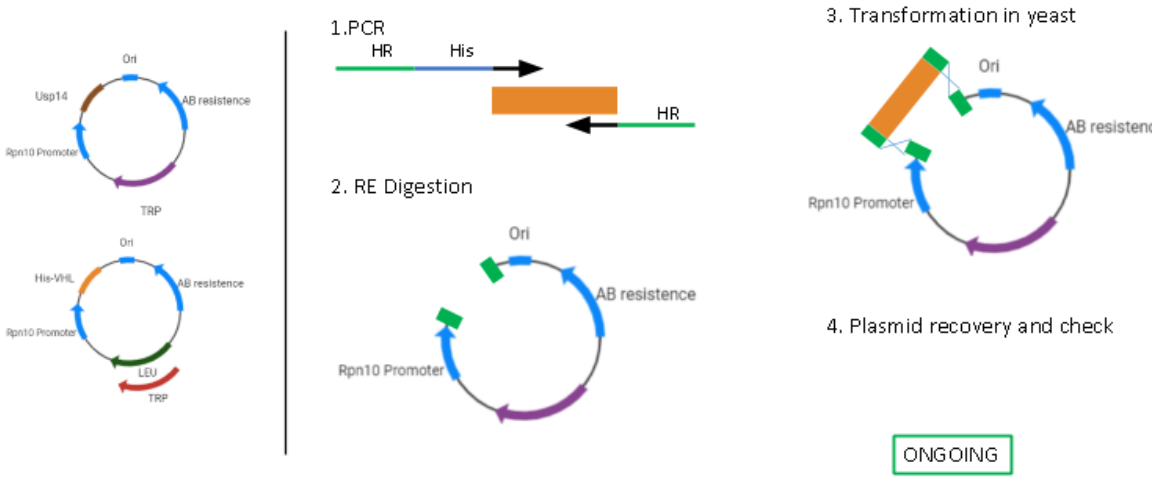


Figure 12; Gap Repair clonation strategy His-VHL. Own source.

3.3 ANALYSIS METHODS

After each procedure, it is necessary to check that the processes have been given correctly and for this the techniques set out below have been needed.

These mainly consist of the Western Blot to check whether or not there is the presence of the cloned protein, DNA gels to know the size of the different fragments obtained and ensure that the intermediate steps have been correct and finally the sequencing, to make sure that the result final is the right one, based on its amino acid sequence.

3.3.1 Western Blot

VHL protein expression was intended to be detected, to achieve it a Western Blot was implemented. This process start with sample preparation, starting by determining how much protein to load and add an equal volume 2X Laemmli sample buffer. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker and run the gel for 1–2 h at 100 V. The polyacrylamide gels that we are using are 4%. Then transfer the protein from the gel to the membrane. For the antibody staining block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer. Then, incubate the membrane with primary antibody in blocking buffer. The primary antibody this project was against VHL. Perform 3 washes of the membrane with TBST to eliminate the excess of primary antibody that has not been bonded. Following, incubate the membrane with the second antibody for an hour, and after repeat the washing with TBST step. Then activate the membrane in methanol and add the substrate that will recognize the secondary antibody. Remove the excess reagent and cover the membrane in transparent plastic wrap. Finally we take to reveal at different times of exposure to observe whether or not there is presence and in what size of

the expected protein. (*General Western Blot Protocol Guidance for Running an Efficient and Accurate Experiment 2 General Western Blot Protocol*, 2020).

3.3.2 Electrophoresis

In order to validate all the procedures, and to be aware if the subsequent steps are followed correctly, DNA validations must be performed trough electrophoresis.
The first step for electrophoresis is to prepare the gel, since they are not very large, nor do we need much separation between bands, we make a standard 1% agarose gel.
In first place, mix agarose powder with 100 mL 1xTAE in a microwavable flask, then microwave it for 45 seconds to dissolve completely the agarose, and level up with water to reach the original weigh of the mixture. Let the agarose cool down, waiting 5 minutes. Add 5 µL of SYBER-SAFE, to observe the DNA. Finally load the sample and run the gel.

PCR	MiniPrep	Doble Digestion	Plasmid Control
5 µL Sample	2 µL Sample	50 µL Sample	1 µL Sample
5 µL H20			
2 µL Orange 6 X	10 µL Orange 1 X	10 µL Orange 6X	10 µL Orange [1x]
12 µL [1X]	12 µL [0,8X]	60 µL [1X]	11 µL

Table 6; Loading volumes depending on what it is being analyzed by electrophoresis

3.3.2.1 Band Purification

Depending on the amount of sample we load on the electrophoresis gel, these bands can be purified to regain the charged DNA. To do this we use the Kit NZYGelPure. The protocol used was the following, in first place transfer to an Eppendorf a fragment of DNA of the agarose gel (100 mg approx.). Add 300 µL of Binding Buffer. Next incubate in 60 ºC for 10 minutes and shale until agarose is completely dissolved. When color of the mixture is yellow load the mixture into the NZYTech spin column placed into a Collection tube and centrifuge for 1 minute (discard the flow-through). Next, add 500 µL of Wash Buffer and discard the flow-through again. Later, centrifuge once more for 1 minute to dry NZYTech spin membrane of residual ethanol. Finally place the NZYTech spin column into a clean 1.5 mL tube and elute in 50 µL of Elution Buffer, incubate it for a minute and centrifuge for 1 minute to elute DNA. (*NZYGelpure* | *DNA Clean-up* | *NZYTech*, 2020.)

3.3.3 Sequencing

Finally when the sample was correctly performed and bacteria colonies were grown there was a need to know what the sequence of the plasmid obtained from the transformation was and also the subsequent comparison between said sequence and the sequence of the theoretical plasmid. To do this, we send Eurofins, which uses the Sanger method, to sequence.
Based on the results of the electrophoresis, it is then decided whether the product can be sent for sequencing or not. For example, if electrophoresis is found to contain unwanted bands, the only band we are interested in can be purified.
The product we send contains between 5 µL and 7.5µL of sample, and 2.5µL of first Forward or Reverse with previous dilution 1:20. The sample volume is determined from the nanophotometer measurement.

The result of the sequencing arrives by mail in the form of different file formats such as Fasta, or .ab1 which contains a lot of information. From this file and with the Chromas program, we can build a chromatogram.
In this type of file each base is represented by a different color, and the quality of said base according to the height of these. The higher the height, the more "intensity" that band has, that is, more chances are that band is the basis it tells us.
Finally with the Benchling program, we can proceed to make an alignment between the obtained sequence and the mounted one in a theoretical way and to detect the miss matches.

3.4 FLUORESCENCE ASSAY

In this experiment, the aim is to measure the fluorescence that a protein emits when ubiquitin is eliminated, thus being able to test the functionality of PROTAC, which is in charge of carrying out this process.
To perform fluorescence assay in first place we prepared the ub-AMC buffer and in parallel thaw proteasomes on ice.

Buffer components	Final concentration
Tris 7.4	50 mM
NaCl	100 mM
EDTA	1 mM
DTT	0.1 mM
BSA	6.7 µL
H2O	To 1000 µL
Ub-AMC (added fresh)	1 µM

Table 7; Ub-AMC Activity Buffer composition for Fluorescence Assay

Once the buffer with the Ub-AMC is prepared, add 90 µl of the buffer into each well. Then, add the calibration curve to B1…B8. Move to the fluorimeter, turn it on and select filters. Add 26s proteasome and put the plate in the fluorimeter. Perform reads every 1 min for 15 min. In this way we are able to know the amount of fluorescence that is emitted over time as the proteasome acts by de-ubiquitinating Ub-AMC and emitting a certain frequency of light. (*Ubiquigent* | *Ubiquitin-AMC*, 2019).

Measure:
Excitation 380
Emission 460

3.5 SCRIPT PYMOL

The pyMOL program has been used to make 3D images of different molecules. First, search the Protein Data Bank (PDB) website for the molecule in question to draw. Once the code with the "Fetch" function is known, the molecule is imported into the program. Selection and changes to the different strings with the "Sele" function and from modifying various parameters. Finally the other molecule is imported in the same way and using the align function, an alignment between both can be obtained. The followed script to obtain the images is present in

4. RESULTS

4.1 PROM Rpn10 ; VHL

VHL binding moiety has been validated for PROTAC, for this reason the aim of the project is to get overexpression of this protein. If the VHL protein can be expressed, the PROTAC could be tested and it could be compounded if they are capable of degrading this protein in a directed way.
The start point are plasmids obtained to get VHL gene thanks to Daniel Kaganovich donation. (Kaganovich et al., 2008).

The obtained plasmids were:

- **BC415:** pESC-elongin B/C bidirectional gal promoter 2 inserts
- **BC416:** pESC- VHL-GFP
- **BC417:** pESC GFP-VHL

Those plasmids were induced under galactose promotor. In previous research, was tried to detect VHL by an Immunoblot, anti GFP.

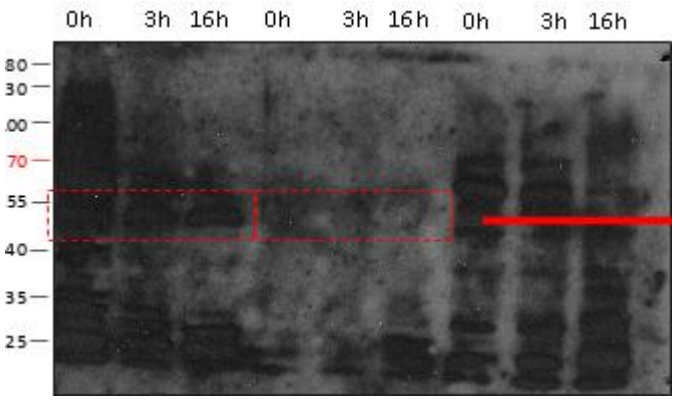


Figure 13; WB anti GFP; Galactose induction for Gal4-ER-VP16 construct

Since this first attempt did not worked as expected, the strategies and objectives, were modified, focusing on a clonation of VHL under the control of the Rpn10 promoter. This is a constitutive promoter which gives moderate levels of expression. We take the opportunity to put a 6His-Tag that will help for detection and posterior purification of the protein. The method that worked and from which the following results were obtained, from the two cloning strategies explained, was the homologous recombination one.

4.1.1 VHL Amplification

Therefore we start a cell cloning, from scratch, starting from the amplification of the VHL gene and 6HIS. The obtaining of this amplified gene, will be the one that will act as an insert for the transformation.

VHL amplification of BC417 plasmid with primers:

- **518:** NDEI-HIS6-VHL FW
  GGAATTCCATATGCATCACCATCACCATCACATGGAGGCCGGGCGGCCGCG
- **520:** VHL-STOP-BAMHI-SPACER Rev
  CGCGGATCCCTAATCTCCCATCCGTTGAT

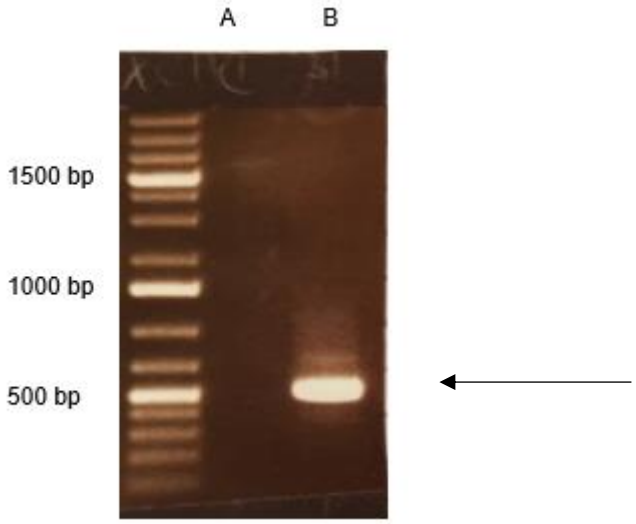


Figure 14; DNA analysis, electrophoresis gel; A: Prom Rpn10 in Prs424, opened plasmid with RE Ndel and BamHI. B: VHL amplification with primers 518 and 520, KOD enzyme; Tm: 42 ºC; For template BC404

In A lane, we see a very faint band, but it is not the one we are using for amplification, therefore, no further analysis is needed. Taking into account the primers length and VHL length, we are expecting a band. In B lane, it is found the expected band, since VHL has 483 bp (base pairs), the total band we expect to see is of (30 + 483 + 16) 529 bp.

4.1.2 USP14 Digestion

Digestion strategy consists on getting rid of USP14 in order to replace it with 6HIS-VHL afterwards, using promRpn10 as its own promotor. Eliminating the gene that comes after Rpn10 promoter, we can substitute it for the gene of interest. The skeleton plasmid is containing promRpn10. Plasmids used for the experiment were:

- BC404: pRS424 PromRpn10 USP14; TRP Auxotrophy
- BC406: pRS425 PromRpn10 USP14; LEU Auxotrophy

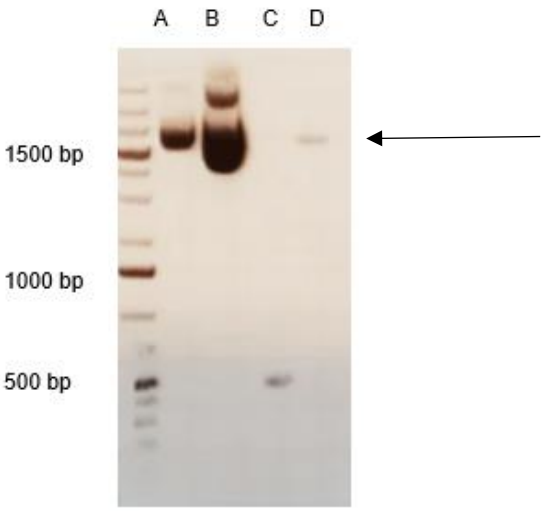


***Figure 15**; A)* Miniprep plasmid BC406 C) VHL PCR D) BC404 digested with BamHII and NdeI restriction enzymes (5 µL charged at a,16,5 ng/ µL concentration.

In D lane, with BC404 digestion we expect to find two bands, one corresponding to the plasmid structure, without USP14, which is around 6,1 kb and a second and smaller band which corresponds to USP14 with 1,5 kb. Even these expected results, there is just the presence of one band, around 1500bp that can correspond to the open plasmid. The USP14 band is not found. We have to take into consideration that the expected band would be around 20% of the total DNA charged (82,5 ng), and it might be that the band is not appreciated because no enough quantity of it is present to be observable in the image.

We can conclude then, that there is presence of this band and digestion has been correctly performed, by using this digested plasmid to further experiments and good results were obtained.

4.1.3 VHL Transformation and Sequencing

Transformation step allows insertion of the vector recently obtained inside the yeast cells, so that they integrate it as their own material. Transfomration is performed into a Wild Type (WT) yeast strain. The strategy for this clonation is gap repaired, based on homologous recombination. For this the cells must be competent, since in this way they will be able to introduce genetic information from abroad. Once done, the cells are sowed in plates rich in YPD medium.

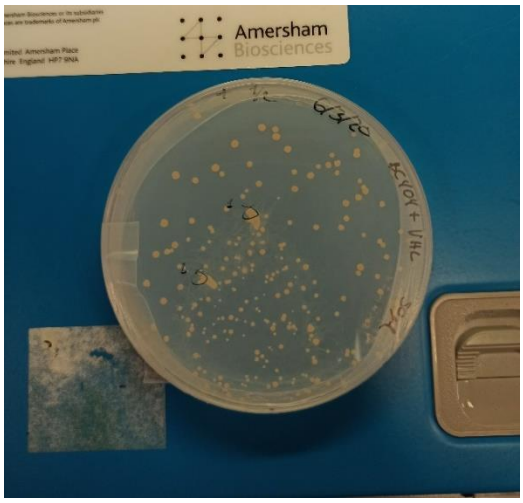


Figure 16; Transformation VHL + plasmid BC404 (Usp14 KO) 50 µL

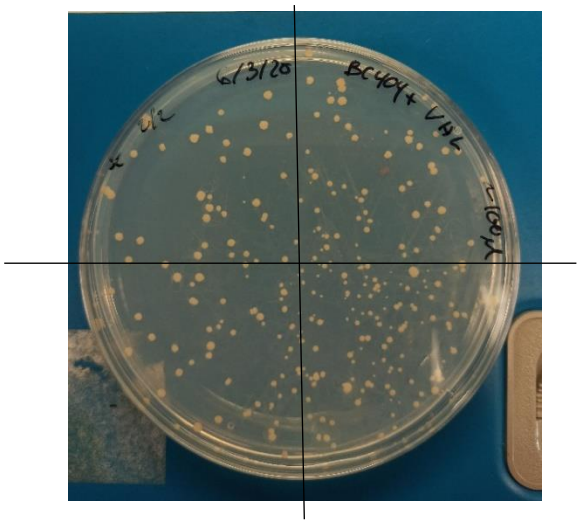


Figure 17; Transformation VHL + plasmid BC404 (Usp14 KO) 100 µL

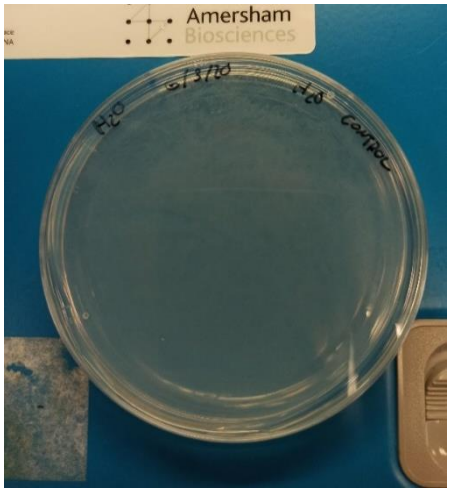


Figure 18; Negative control Transformation with H20

Transformation is correctly performed, we find several colonies that are able to grow as the result of VHL and BC404 (pRS424 PromRpn10 USP14; TRP Auxotrophy) transformation. The number of colony forming units in 100 µL is about 232 colonies.

4.1.4 Sequencing

In parallel, cloning was also carried out from the BC406 plasmid, remember that the difference between both resided in auxotrophy. The plasmid with which the sequencing was carried out was BC406 with LEU auxotrophy.

Once the first transformation was performed, to amplify this vector and to sequence it, from those colonies, a bacteria transformation was accomplished.

In order to detect whether this colonies are the correct ones, and to determine if the sequence obtained is the one that we expected and corresponds with the theoretically vector expected, sequencing is needed.

First, we design computationally the expected plasmid, the theoretically one, if transformation would have been correct, that will be used later to align the sequence.

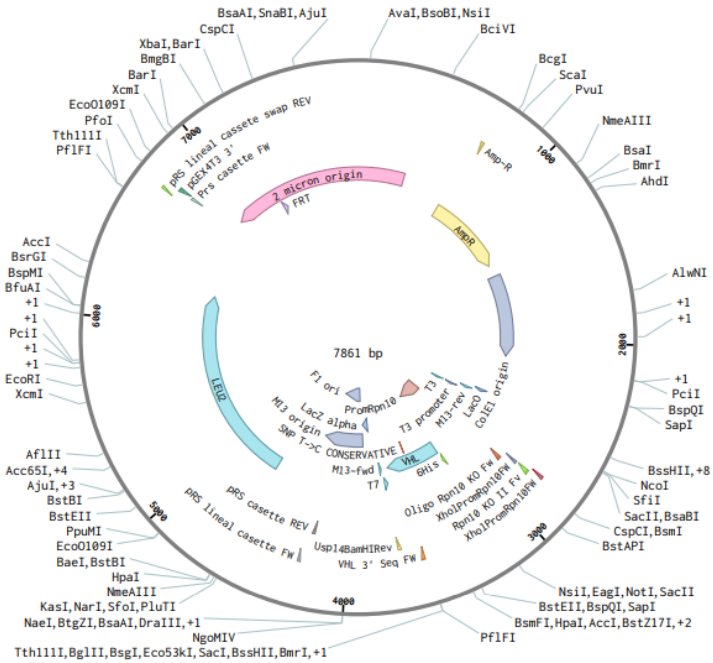


Figure 19; Construct PromRpn10-His-VHL Prs424 LEU (7861 bp) in Benchling

Sequencing is performed in both directions and to get a further accurate and wider result, we perform sequencing of two different regions, therefore with two different primers.

- **Seq ID 08HG83:** #31 Oligo Rpn10 KO FW
- **Seq ID 08HG84:** #517 VHL; Seq FW

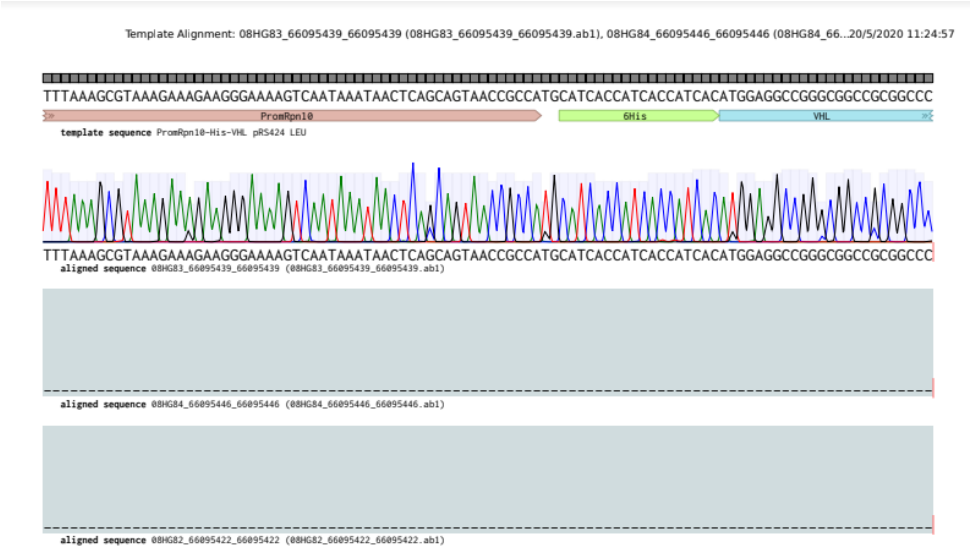


***Figure 20*;** Alignment between template sequence PromRpn10-His-VHL in Prs424 LEU and the sequencing

In this alignment performed, it is found the reference sequenced based on the plasmid Rpn10-His-VHL in PRS424 with LEU auxotrophy align with the sequenced data. The second row corresponds to the alignment performed with the Seq ID 08HG83 and the third one with the Seq ID 08HG84, obtaining this way a wider range sequenced.

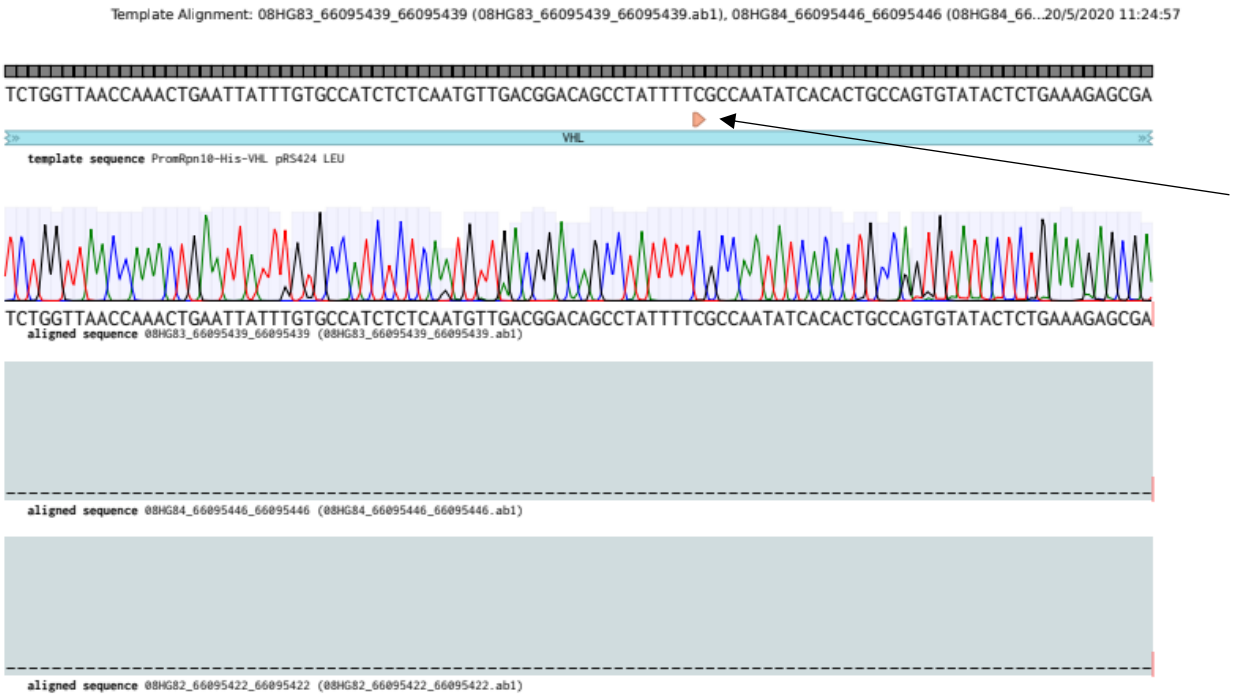


Figure 21; Aligment between template sequence PromRpn10-His-VHL in Prs424 LEU and the sequencing

A miss match is present in the sequencing, between the template sequence and the first align sequence. In the template there was a Thymine (changed in the alignment image for the corresponding C) base but in the second align sequence there is a Cytosine. The first miss match present changes a Thymine for a Cytosine, and we can conclude that this change in bases is not implicated in a change of AA, it is a silent mutation, since both codons codify for the same amino acid, Phenylalanine (Phe).

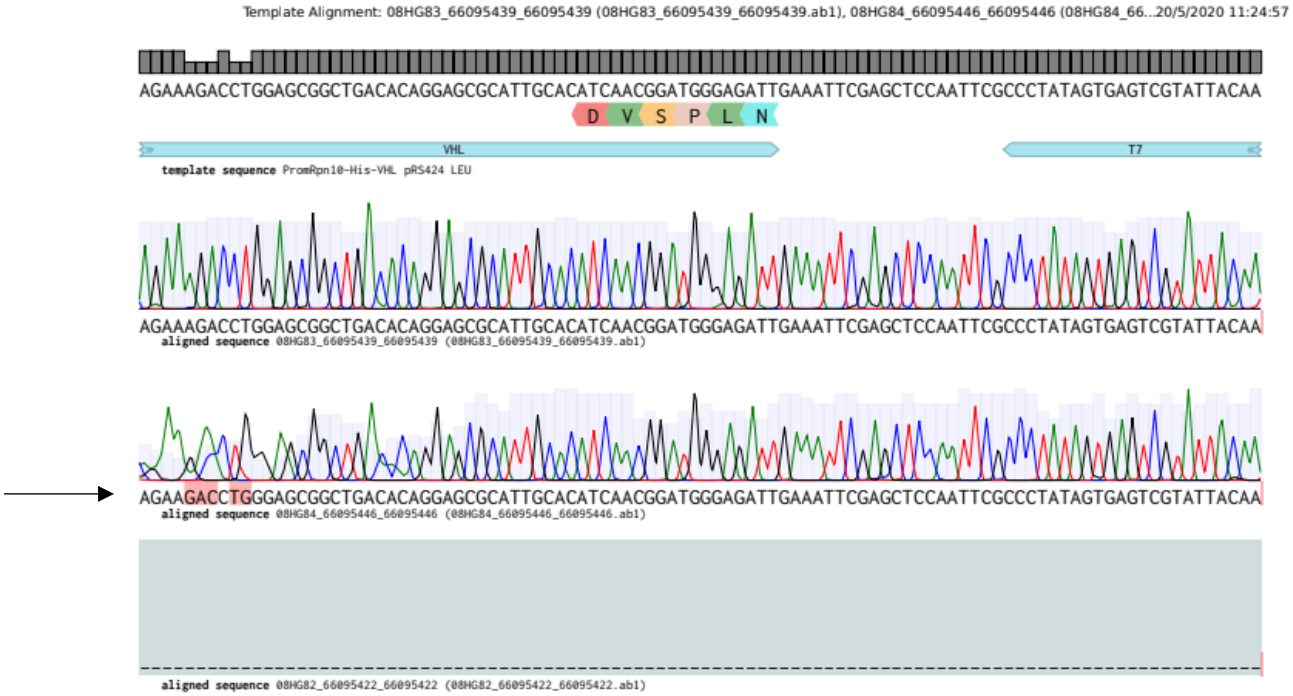


***Figure 22**;* Aligment between template sequence PromRpn10-His-VHL in Prs424 LEU and the sequencing

Another mismatch is observed in the second image. On the template sequence and in the first align sequence there is an AGA codon, while in the second aligned sequence there is a GAC one. A second mismatch is found, with CCT codon in both template and first aligned sequence but with CTG codon in second aligned sequence.

AGA codon is codifying for Arginine AA while GAC is codifying for Aspartic Acid. Asp is an acid AA while Arg is a basic one. CCT codifies for a proline which is a big AA with cyclic lateral chain and CTG for Leucine which is an aliphatic non-charged amino acid. As the changes from one codon to another transform them into amino acids with different characteristics, I can conclude that this is a sequencing error since, in the first aligned sequence, it was correctly observed in correspondence with the template. The reason why it can appear a miss-match in this position is because it is the beginning of Seq ID 08HG84 sequencing, and it is known that the early bases on sequencing can present errors.

Another concept that can provide more validity to our argument is that behind the colors that correspond to each base, there are some light blue bars, almost transparent, that correspond to quality. Meaning how likely is it that the sequenced base is correct. While in most bases we see a high quality greater than 60, in bases where there are miss matches, we see that the quality is low, that is to say over 20, this may indicate that it is a sequencing error.

4.1.5 Selection Marker

Once we have the PromRpn10-6His-VHL construct with LEU marker, we want to make a marker change to be able to use the ΔUbp6 strains. ΔUbp6 strains have a URA auxotrophy selection marker, so if we want to grow them, we will have to modify the selection marker.
In order to do this we will use the HR technique (Homologous Recombination).

- **S67 WT ; s590:** The yeast strains that we are using are as explained before are ΔUbp6, and they are also URA free. For this reason, those yeasts won't be able to grow without URA in the medium.
- **s592:** Those yeast strains are ΔUbp6 and they are also ΔPDR5. It is inserted Kan Resistance in the locus of PDR5. PDR5 is a plasma membrane ATP-binding cassette (ABC) transporter involved in resistance to multiple drugs; facilitates drug removal from the cell. It allows certain resistance to a non-specific drug. If this gene is inhibit in our strains, PROTAC won't be eject from the cell. (Balzi et al., 1994)

Two PCR to amplify are performed:

- A PCR:

Template:
prs426 (It is an empty pRS with URA marker)
Primers:
#479 pRS casette FW cctctgacacatgcag
#478 pRS casette REV ctcaaccctatctcggtc

- B PCR:

Template:
PromRpn10-6His-VHL with LEU marker plasmid
Primers:
480 pRS linear cassette HRswap REV ctgctcccggcatcc
481 pRS linear cassette HRswap FW ccaataggccgaaatcg

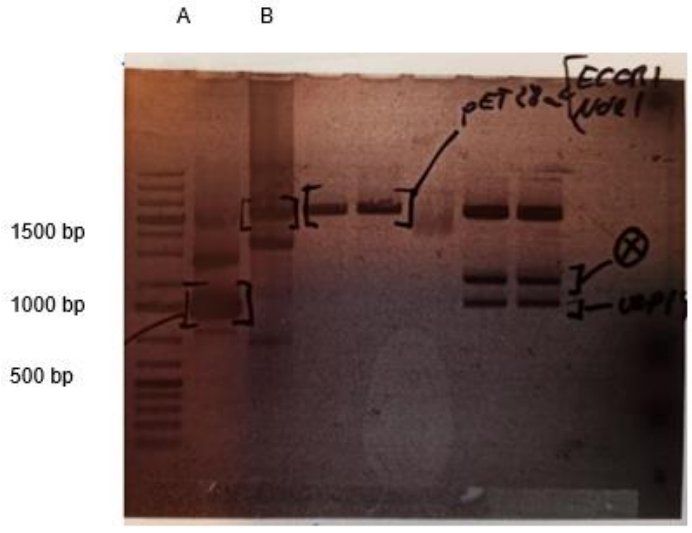


Figure 23; DNA electrophoresis Gel, corresponding to both PCR amplification. A lane: PCR amplification prs426. Primers: 479 and 478. Tm= 42º C. B lane: PromRpn10-6His-VHL amplification. Primers 480 and 481. Tm= 42 ºC. KOD polymerase enzyme.

This amplification to use gap repair method we can observe thanks to this result, that is correctly performed. In A lane, we were expecting a band around 1000 bp which corresponds to URA marker. In B lane, we were expecting a band around 1500 bp, wichi corresponded to the plasmid, but without LEU marker.

4.1.6 Protocol Development

Cell transformation was performed by two different strategies, homologous recombination, based on gap repaired (explained before) and basic clonation. With this second strategy we didn't obtain good results, for this reason we wrote and proposed protocol an improved and detailed protocol of this cloning strategy, for use in future experiments.

- Vector Digestion

First digest the vector, with the desired restriction enzymes. To choose them, pass vector and insert to nebcutter 2 and look for those with List 1 cutters for the vector and 0 cutters for the insert. Compare with the list of enzymes in the lab. Search the Neb website for the most suitable Buffer and choose the one that gives 100% efficiency. To digest we load 1 µg of DNA and up to a volume of 50 µL. To check the efficiency of both restriction enzymes, we also do two partial digestions, with half the volume (25 µL). Incubate at 37 ºC 1h / 1h: 30 Add 1 µL of alkaline phosphatase, only to the digestion of the vector and allow to incubate at 37 ºC for another 15 minutes. Load all three samples on ice and run to check that the bands are the right ones. (We load the whole digestion and cut aside).

- Insert Amplification

To perform the PCR we use 1 µL of the enzyme KOD. Final volume of 50 µL Make 1:20 dilution of the first both Fw and Rev. Prepare the thermocycler before adding the enzyme (the KOD), adjust the Melting Temperature according to the first selected, choose 5 ºC less than the lower of the two. Repeat 30 cycles. Finally, add the enzyme and apply PCR. To validate that the insert is correct and expected, load 5 µL of the PCR into a gel and check if the band corresponds to the desired size. Purify both the gel containing the digested vector and the PCR and check on a gel that they are correct.

- Ligation

To bind vector and insert use the enzyme ligase. Put between 10 - 100 ng of vector and insert to maintain a 3: 1 ratio with the vector. Final volume of 20 µL.
As we use fast ligatures the time for ligation varies between 5 - 15 min at room temperature 22 ºC. Remove an unbound sample control, and a bound sample control (after 15 minutes). Run gel electrophoresis.

- Transformation

5 µL of binding to the competent cells.
Positive control of closed plasmid transformation (1µl dil 1: 100 of puc19)
Negative control (use 5 µL of a ligation where we have not added insert) in those that are not resistant to the antibiotic.

Load all the transformation into the plates (50 µl on one plate and 150 µl on another, a total volume of 200 µl).
Leave at 37 ºC (E.coli) overnight.

Two methods to check that the connection is correct are:
- Put 5ml preculture in LB + Antibiotic, do Miniprep + PCR or control digestion.
- Control PCR directly using a "pipette tip" template. Choose primers (in this case primers already existing in the laboratory) to amplify a region of this new vector that includes both part of the original vector (the promoter) and the insert. Do a PCR to amplify it, calculate the expected band size and check if the digestion is correct.
Put a positive control where the PCR is done by the DNA of the original vector and the first are the right ones for it. Final volume 25 µL.

Another check to make sure that there is no old insert that we intended to remove, is to choose an enzyme that cuts through the digested part and therefore removed from the vector and does not cut in the BackBone, nor in the insert of this way we will make sure to select only those that have done the digestion correctly.

Transformation Verification: Pre-cultivate with the colonies resulting from planting, purify them from Miniprep Kit and calculate their concentration. Those that have tested positive in either control digestion or PCR are sequenced. We make Miniprep and before sending they run on an ice. To send to sequencing put 5µl of 1:40 first + 5µl of Miniprep at [40-100] ng / ul. If the MP is more concentrated, we can dilute it. Send to sequence.

4.2 FLUROESCENCE IN VITRO ASSAY

Bibliography has evidences that USP14 is the homologous domain of Ubp6 in Homo Sapiens. Having similar structure and similar function. Ubp6 (USP14 in mammals) is an ubiquitin-specific protease (USP). Occupancy of those DUBs by an ubiquitin conjugate leads to ATPase stimulation, coupling deubiquitination. Later, ubiquitinated loosely folded proteins, (once they are after bound to the 26 S), interact with Ubp6/USP14 to activate ATP hydrolysis and enhance their own destruction. (Peth et al., 2013). Previous experiments had been performed in order to substitute yeast proteasome Ubp6 by mammalian USP14. To test if this substitution has been correct and if USP14 can actually act and accomplish the same function of Ubp6 in proteasome we prepare a fluorescence assay in vivo, that will help us phenotypically. For this test, we use ubiquitine AMC, which is a fluorogenic substrate that increases fluorescence as a result of the cleavage between the C-terminal Glycine and AMC, creating Ubiquitin and de-quenched AMC.(*Ubiquigent | Ubiquitin-AMC*, 2020)

We expect the follow results in three conditions, Wild Type, ΔUbp6, ΔUbp6:USP14 :

- WT: A wild type proteasome, in contact with Ub-AMC, the DUB Ubp6 will lead free AMC leading fluorescence.
- ΔUbp6: There won't be fluorescence signal, because not deubiqutination is performed
- ΔUbp6:USP14: Should act as a Wild Type

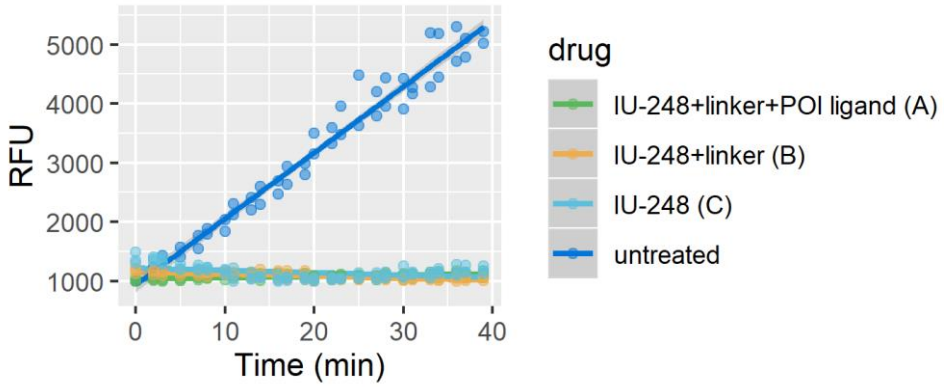


Figure 24; Interaction of initial synthesized molecules with the proteasome. Ub-AMC assay: USP14-26S was purified according to lab procedures, 4nM purified 26S was incubated with 0,5 µM Ub-AMC and 100 µM drug candidate. The deubiquitinase activity was monitored in a plate fluorimeter (Excitaton 380 nm, Emissio:460nm). USP14 inactivation is taken as a measure of efficient drug interaction with the proteasome.

In this graph we observe how a purified 26S proteasome with USP14 acts in contact with Ub-AMC. The several drugs tested correspond to different ligands of USP14.

- Untreated: In the untreated one, USP14 is free and therefore it has DUB's activity showing fluorescence, as if Ubp6 was present.

- IU-248: IU-248 is a described USP14 inhibitor. IU-248 binds USP14 inhibiting its function and stopping its deubiquitinase activity. Therefore, there is no increase in the fluorescent signal.
- IU-248 + linker: It has the same effect as explained before, but in this case the inhibitor is united to the ligand of the PROTAC
- IU-248+ linker + POI ligand: The effect is the same because of USP14 inhibition. In this experiment it is tested the union of the proteasome to the IU inhibitor plus the linker and the ligand of the target protein (to be further degraded) of PROTAC.

4.3 3D PYMOL IMAGES

In this section it has been proceeded to create 3D images that allow us to structurally understand some of the molecules that have been worked on the most during this project. Pymol is a science-oriented computer tool, to help those who need to know the arrangement of molecular structures of different substances.

4.3.1 Ubp6:USP1

In this first section, the structure of the proteasome has been designed, since it is in charge of degrading proteins, highlighting the domain of Ubp6 with which so much work has been done and joined to its USP14 counterpart. With this image, it was intended to structurally verify the homology between the two.

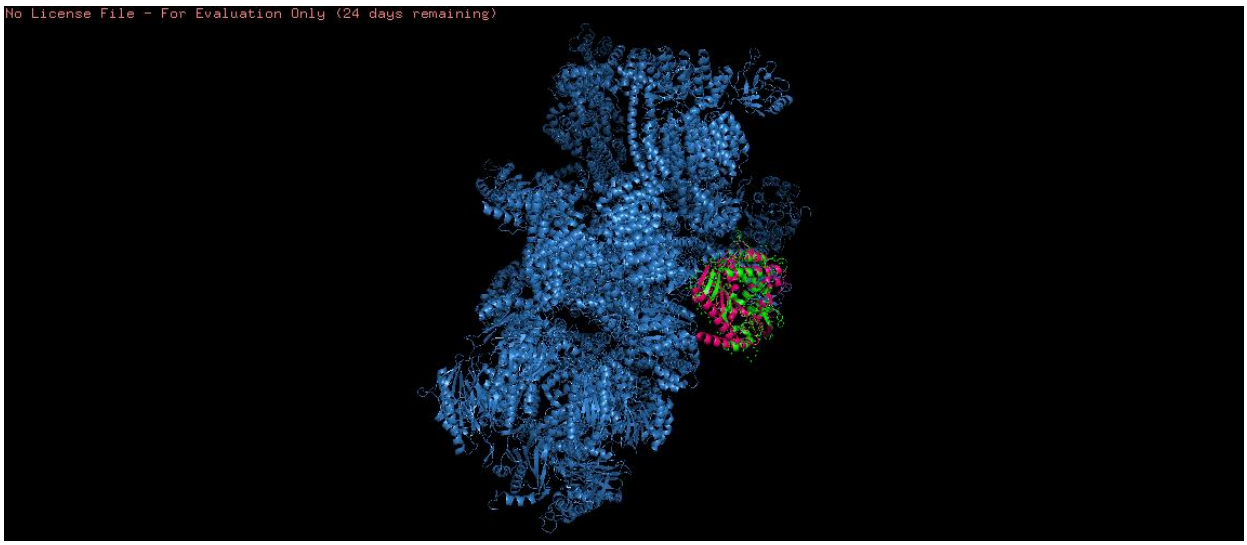


Figure 25; 26S Proteasome, Ubp6: pink, USP14: green by pyMOL Image I. Own source.

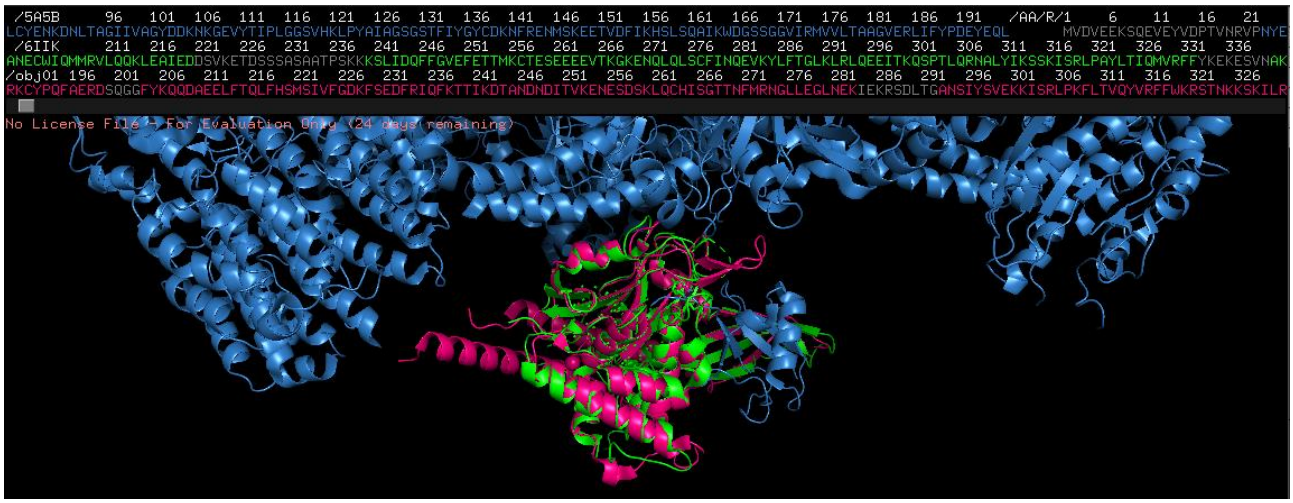


Figure 26; 26S Proteasome, Ubp6: pink, USP14: green by pyMOL Image II. Own source.

In these images it is appreciable the whole proteasome 26S, which is marked in blue, with its Ubp6 subunit marked in pink. In parallel USP14 is aligned to Ubp6. In this way it can be verified that Ubp6 and USP14 are homologous as they can be properly aligned with each other.

4.3.2 VHL

In this image the VHL protein has been made marking in red the own protein, in purple the chain corresponding to the Elongin B and the yellow one is the Elongin C, can be observed therefore that for a correct expression of VHL the simultaneous expression is required of elongins B and C. In parallel, as VHL will be the target protein to be degraded, bibliographic research has been done on a possible inhibitor of VHL in order to bind to it. It has been found that this inhibitor would bind to the outermost area of this molecule, and this would be bound to PROTAC by a liker. A possible inhibitor is VH298, that stabilizes HIF-α and elicits a hypoxic response via the blockade of the VHL. (Buckley et al., 2012)

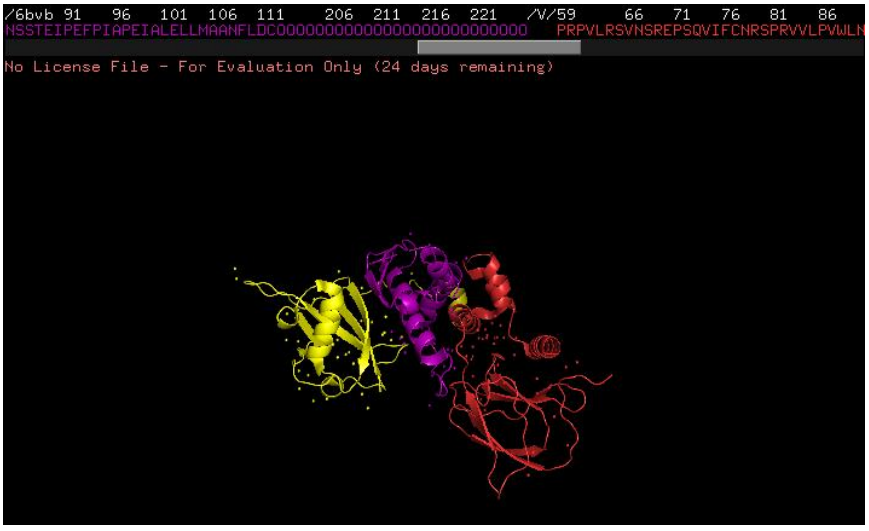


Figure 27; VHL protein: red , Elo_B: purple, Elo_C: yellow by pyMOL Image I. Own source.

5. CONCLUSION

1. VHL can be clonated under PromRpn10.

2. Although both cloning techniques have been applicable, as long as an improved cloning protocol is not achieved, the gap repair method offers more effectiveness and is simpler for a cloning process.

3. The homology between the Ubp6 subunits in yeast and USP14 in mammals, tested from both phenotypic and computational assays, can be stated.

6. LIMITATIONS

The implementation of this project has been cut and limited due to the global pandemic caused by the SARS-CoV-2 virus. Due to the quarantine situation, the practical and face-to-face hours in the laboratory to carry out the different experiments have been truncated. That is why both the approach and the initial objectives of the project have been modified and consequently reduced.
As explained on the objectives this task is a small piece of a bigger project involving the development of an independent-E3 PROTAC molecule.
Other directions in which I intended to orient my task was in the in vitro expression of the VHL-elongin B and elongin C enzyme complex, since it is known that for VHL to be stable and function correctly, it has to be co-expressed with elongins B and C. Using laboratory protocols (endowed by Dr. Judith Frydman, Stanford) we intended to express this protein in vitro, which would be the degradation target of PROTAC.

Second, as also explained above, Ubp6 has been replaced by its USP14 mammalian counterpart, with the aim of using the humanized proteasome as a tool to validate PROTAC molecules. So far we have been able to check that the molecules bind the proteasome side, as seen in the Ub-AMC fluorescence assays. It is planned to further validate PROTAC with USP14 26S *in vitro* protein-of-interest degradation experiments.
Furthermore, in previous experiments it had been observed that when purifying the proteasome with USP14 and trying to detect USP14 via western blotting, this domain was not detected, although phenotypically with other tests, it could be observed that it fulfilled its function. This partial dissociation may happen in the purification process. For this reason, another objective of this project was to focus on the binding of USP14 to the proteasome and to be able to detect it.

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whEhGbmNdjS03Jc97Sg4EhoCcEsQAvD_BwE

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8. ANNEX

8.1 Script Ubp6:USP14 Image

```
PyMOL(TM) 2.4.0 - Incentive Product
 Copyright (C) Schrodinger, LLC


PyMOL>fetch 5A5B
TITLE     Structure of the 26S proteasome-Ubp6 complex
 ExecutiveLoad-Detail: Detected mmCIF
 CmdLoad: ".\5a5b.cif" loaded as "5A5B".
PyMOL>sele Ubp6, chain 8
 Selector: selection "Ubp6" defined with 3056 atoms.
 Executive: Colored 85300 atoms and 1 object.
 Executive: Colored 85300 atoms and 1 object.
 Executive: Colored 3056 atoms.
 You clicked /5A5B/H/8/TYR`494/CA
  You clicked /5A5B/H/8/GLU`169/CA
 Selector: selection "Ubp6" defined with 3056 atoms.
 Executive: Colored 3056 atoms.
PyMOL>fetch 6IIK
TITLE     USP14 catalytic domain with IU1
 ExecutiveLoad-Detail: Detected mmCIF
 CmdLoad: ".\6iik.cif" loaded as "6IIK".
 Setting: seq_view set to on.
 Setting: seq_view set to off.

 Match: read scoring matrix.
 Match: assigning 798 x 415 pairwise scores.
 MatchAlign: aligning residues (798 vs 415)...
 MatchAlign: score 576.500
 ExecutiveAlign: 373 atoms aligned.
 ExecutiveRMS: 21 atoms rejected during cycle 1 (RMSD=3.27).
 ExecutiveRMS: 20 atoms rejected during cycle 2 (RMSD=1.62).
 ExecutiveRMS: 17 atoms rejected during cycle 3 (RMSD=0.95).
 ExecutiveRMS: 12 atoms rejected during cycle 4 (RMSD=0.73).
 ExecutiveRMS: 6 atoms rejected during cycle 5 (RMSD=0.64).
 Executive: RMSD =    0.608 (225 to 225 atoms)
 Executive: object "aln_6IIK_to_obj01" created.
 Executive: Colored 5883 atoms and 1 object.
 Executive: Colored 3056 atoms and 1 object.
 Executive: Colored 1 object.
 Executive: Colored 1 object.
 Executive: Colored 5883 atoms and 1 object.
PyMOL>rock
PyMOL>rock
PyMOL>png C:/Users/mjart/Documents/26S Ubp6Usp14.png, 0, 0, -1, ray=0
 ScenePNG: wrote 1146x494 pixel image to file "C:/Users/mjart/Documents/26S Ubp6Usp14.png".
PyMOL>png C:/Users/mjart/Documents/26S Ubp6Usp14 (2).png, 0, 0, -1, ray=0
 ScenePNG: wrote 640x515 pixel image to file "C:/Users/mjart/Documents/26S Ubp6Usp14 (2).pn
g".
```

```
PyMOL>png C:/Users/mjart/Documents/26S Ubp6Usp14 BONA1.png, 0, 0, -1, ray=0
 ScenePNG: wrote 1146x494 pixel image to file "C:/Users/mjart/Documents/26S Ubp6Usp14 BON
A1.png".
 You clicked /6IIK/F/B/HOH`719/O
 Selector: selection "sele" defined with 126 atoms.
 Clean: Cleaning 378 atoms.  Please wait...
 Clean: Finished. Energy = -1352.65
PyMOL>png C:/Users/mjart/Documents/26S Ubp6Usp14 BONA2.png, 0, 0, -1, ray=0
 ScenePNG: wrote 1146x494 pixel image to file "C:/Users/mjart/Documents/26S Ubp6Usp14 BON
A2.png".
 You clicked /6IIK/B/B/ILE`174/CA
Setting: seq_view set to on.
```

8.2 Script VHL 3D Image

```
 PyMOL(TM) 2.4.0 - Incentive Product
 Copyright (C) Schrodinger, LLC


Detected 4 CPU cores.  Enabled multithreaded rendering.
HEADER    GENE REGULATION                         12-DEC-17   6BVB
TITLE     CRYSTAL STRUCTURE OF HIF-2ALPHA-PVHL-ELONGIN B-ELONGIN C
COMPND    MOL_ID: 1;
COMPND   2 MOLECULE: VON HIPPEL-LINDAU DISEASE TUMOR SUPPRESSOR;
COMPND   3 CHAIN: V;
COMPND   4 SYNONYM: PROTEIN G7,PVHL;
COMPND   5 ENGINEERED: YES;
COMPND   6 MOL_ID: 2;
COMPND   7 MOLECULE: ELONGIN-B;
COMPND   8 CHAIN: B;
COMPND   9 SYNONYM: ELOB,ELONGIN 18 KDA SUBUNIT,RNA POLYMERASE II TRANSCRI
PTION
COMPND  10 FACTOR SIII SUBUNIT B,SIII P18,TRANSCRIPTION ELONGATION FACTOR B
COMPND  11 POLYPEPTIDE 2;
COMPND  12 ENGINEERED: YES;
COMPND  13 MOL_ID: 3;
COMPND  14 MOLECULE: ELONGIN-C;
COMPND  15 CHAIN: C;
COMPND  16 SYNONYM: ELOC,ELONGIN 15 KDA SUBUNIT,RNA POLYMERASE II TRANSCRI
PTION
COMPND  17 FACTOR SIII SUBUNIT C,SIII P15,TRANSCRIPTION ELONGATION FACTOR B
COMPND  18 POLYPEPTIDE 1;
COMPND  19 ENGINEERED: YES;
COMPND  20 MOL_ID: 4;
COMPND  21 MOLECULE: HYPOXIA-INDUCIBLE FACTOR 2 ALPHA;
COMPND  22 CHAIN: H;
COMPND  23 ENGINEERED: YES
 ObjectMolecule: Read secondary structure assignments.
 ObjectMolecule: Read crystal symmetry information.
 CmdLoad: "" loaded as "6bvb".
PyMOL>sele VHL, chain V
 Selector: selection "VHL" defined with 2352 atoms.
PyMOL>sele Elo B, chain B
 Warning: Invalid characters in 'Elo_B' have been replaced or stripped
 Selector: selection "Elo_B" defined with 1633 atoms.
```

PyMOL>sele Elo_C, chain C
Selector: selection "Elo_C" defined with 1436 atoms.
Executive: Colored 2352 atoms.
Executive: Colored 1633 atoms.
Executive: Colored 1633 atoms.
Executive: Colored 2352 atoms.
Executive: Colored 1436 atoms.
You clicked /6bvb//V/GLN`96/CA
Selector: selection "sele" defined with 17 atoms.
You clicked /6bvb//V/PRO`97/CA
Selector: selection "sele" defined with 31 atoms.
You clicked /6bvb//H/MET`535/CA
Selector: selection "sele" defined with 17 atoms.
You clicked /6bvb//V/TYR`98/CA
Selector: selection "sele" defined with 2352 atoms.
You clicked /6bvb//H/PRO`534/CA
Selector: selection "sele" defined with 2549 atoms.
You clicked /6bvb//H/PRO`534/CA
Selector: selection "sele" defined with 2352 atoms.
You clicked /6bvb//V/GLY`127/CA
Selector: selection "sele" defined with 0 atoms.
You clicked /6bvb//H/PRO`534/CA
Selector: selection "sele" defined with 197 atoms.
You clicked /6bvb//B/GLY`76/CA
Selector: selection "sele" defined with 1633 atoms.
You clicked /6bvb//B/GLY`76/CA
Selector: selection "sele" defined with 0 atoms.
Executive: Colored 1633 atoms.
Executive: Colored 1633 atoms.
You clicked /6bvb//H/GLY`537/CA
Selector: selection "sele" defined with 197 atoms.
Setting: seq_view set to on.
PyMOL>fetch 1vcb
TITLE THE VHL-ELONGINC-ELONGINB STRUCTURE