Introduction

Targeted protein degradation has become one of the most promising disease treatment strategies. One method for inducing degradation is to use proteolysis-targeting chimeras (PROTACs). PROTAC is a bifunctional chemical molecule with different ligands at either end connected by a linker, one ligand that binds to E3 ligase and another to an intracellular protein of choice. PROTAC can use the cell's own ubiquitin protease system (UPS) to target and induce protein degradation. Such molecules bind both the E3 ubiquitin ligase and intracellular proteins, resulting in polyubiquitination of the target protein by recruiting it to the vicinity of the E3 ubiquitin ligase. Ubiquitinated protein is eventually degraded by the proteasome[2].

G Protein-Coupled Receptors (GPCRs) are the largest family of membrane proteins in the mammalian genome. They are widely distributed in organs and tissues such as the central nervous system, immune system and retina, and participate in the development and normal function of the body. If the regulation of the related intracellular pathway is abnormal, a series of diseases will occur. Because of the ubiquity and importance of GPCR expression in humans, this family has become an important drug target[1]. As GPCRs are sensing extracellular hormones, their ligand binding site is exposed to the extracellular space, and majority of drugs acting on GPCRs bind to this site. In certain conditions, such as cancer, specific GPCRs are overexpressed, and their targeted degradation by PROTACs is a very promising therapeutic strategy. Modifying existing ligands by attaching a E3 ligase recruitment moiety to existing ligands is good strategy to develop GPCR PROTACs.

This brings a problem: Can the cell recognize and degrade membrane proteins when the PROTAC is recognizing the extracellular side? And if yes, does it degrade during the production process (i.e. in the ER) or at the cell surface? We addressed this question by overexpressing a recombinant protein, a GPCR with a Halo-tag on either the extracellular or intracellular side and targeting it with a Halo-PROTAC-E[2]. Halo-PROTAC-E is capable of degrading intracellular HaloTag (a modified haloalkane dehalogenase that covalently reacts with hexyl chloride tags) and the mechanism is established[2]. We engineered a Halo-tag on the GPCR (CB2) N-terminal (figure 2). In this experiment, whether degradation occurs on the cell membrane or on the ER membrane is investigated. Whether the degradation can happen after PROTAC binds to extracellular HaloTag (N-terminal linkage) is studied as well.

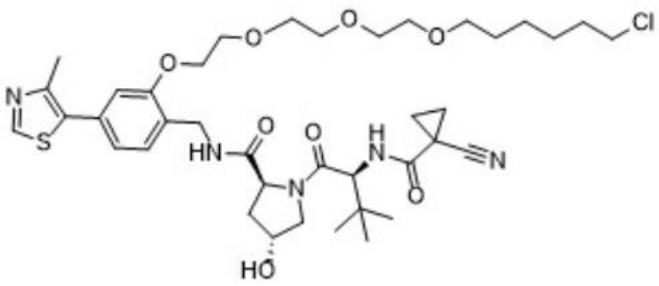

Figure 1. structure of HaloPROTAC-E

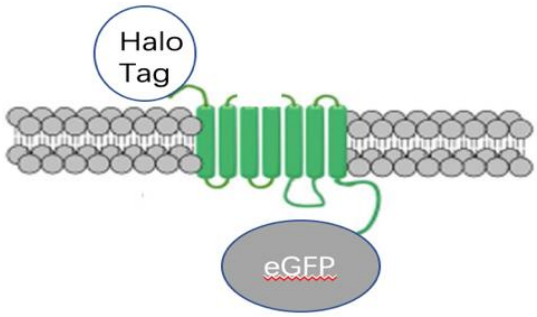

Figure 2. structure of the membrane and the N-terminal Halo-tag, CB2 and eGFP

Methods

Cloning

Two fragments (Halo tag was used as an insert and the backbone contained eGFP and CB2) are made using PCR. 8.75µL of mQ, 1.25µl of Plasmid 223, 1.25µL of primer 198, 1.25µL of primer 195 and 12.5µL of 2x Primestar polymerase were mixed for backbone PCR. 3.5µL of mQ, 0.5µl of plasmid 220, 0.5µL of primer 196, 0.5 µL of prime 197 and 5µL of 2x Q5 polymerase were mixed. A touch-down PCR protocol with a decrease of annealing temperature of 0.5 °C per cycle was used. The vector was annealed from 71 ℃ and the insert was annealed from 68 ℃. After PCR, the two fragments were checked on an agarose gel. The template DNA was digested with DpnI (0.1µl for insert and 0.4 µL for vector) at 37 °C for 3 h, then at 16 ºC for 11 hr. MinElute Reaction Cleanup Kit (Qiagen) was used for reaction clean up. A Gibson assembly at 55 °C for 15 min followed by 1 h at 50 °C with 0.37 µL of vector, 0.76 µL of insert, 1.37 µL of mQ and 7.5 µL of assembly mix was used. Transformations were done with 4 µL assembly product and 60 µL chemically competent Escherichia coli Top10 cells. Transformed cells were plated on lysogeny broth (LB) agar plates for 20 hours. 5 mL Luria Broth, 5 µL carbenicillin and a colony were then added into a 14 ml cell culture tube and incubated 16-18 hours at 220 rpm and 37 ºC. The cultures were then spun down for 10 min at 4122g in ThermoFisher Multifuge X3 F3. The plasmid DNA is isolated using a QIAprep Spin Miniprep Kit (Qiagen) with elution in 50 µL mQ. 700 ng samples were then sent for sequencing.

Cell culture

Transfection

HEK-293TRs cells were maintained in DMEM media supplemented with 10% DPBS at 37 ℃ and 5% CO_2. 80 µL OptiMEM and 100 ng plasmid 222 (pcDNA4/TO-Halo-CB2-eGFP)were added in a vial and 80 µL OptiMEM and 300 ng PEI were added in another vial. 20 µL OptiMEM and 100 ng DNA were added in a vial and 20 µL OptiMEM and 300 ng PEI were added in another vial. HEK293TR cells were trypsinised, discard supernatant and then resuspend in 9 mL DMEM media. Around 50000 cells per well in 100 µL media were transfected with the DNA/PEI mix and plated in a 96-well plate and incubated at 37 ℃ and 5% CO_2 until to ~85 % confluency (48 h).

SDS-Page gel

Cells were treated with 2 µL 30uM HaloPROTAC-E for 0, 2, 4 or 6 hours. Media was aspirated and transferred into a microcentrifuge tube. Tubes were then spinned down at 16.9 kg, 4 ℃ for 10 minutes. After removing the supernatant, 2.5 µL laemlli buffer and 7.5 µL DPBS was added. 5

µl was loaded into each well of a TruPAGE 4-20% mini-gel (Thermo Fisher). The gel was run for 40 min at 100 mA.

In-gel fluorescence

The gel was first washed 3x in DPBS and imaged on a Typhoon laser scanner (Amersham) using the Cy2 filter set to observe eGFP-containing bands. The gel was then fixed and stained with SYPRO red protein gel stain (thermoFisher) in 7.5% acetic acid overnight, washed 3x in DPBS and imaged using the Cy5 filter set to observe the total protein content of each well.

Analysis imaging data

Images were obtained from a previous student. Roughly, these were produced using the same transfection method, and exposure to Halo-PROTAC-E as described above and then followed by staining using DIL stain (ThermoFisher) for the plasma membrane, ER cytopainter (Abcam) for the ER and bisBenzimide H 33342 trihydrochloride for the nuclei.

The images were analysed using Cellprofiler. To determine colocalization, the objects within the image must be identified. By setting membrane as channel c=0, bright field c=1, receptor (CB2 eGFP) c=2 and DNA c=3. The two-class otsu thresholding was used.

Results

Cloning of eGFP-CB2-Halo

The cloning was nearly successful except there is a stop codon (TAG) in the insert.

Figure 3. from left to right 224-Vector, 224-insert and Gene Ruler 1kb

The DNA gel matches the length of backbone (7179 bp) and insert (892 bp).

Quantification of degradation of Halo-CB2-eGFP

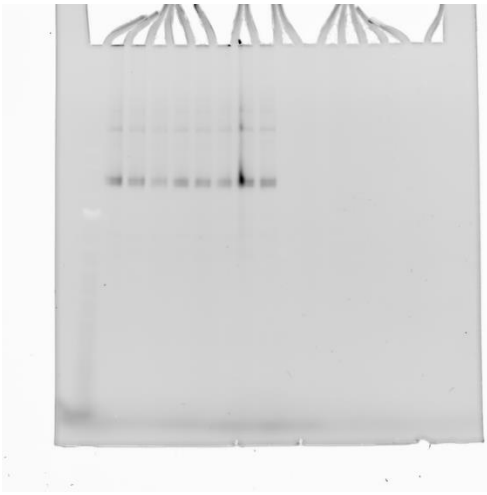

Figure 4. SDS-PAGE gel of Halo-CB2-eGFP

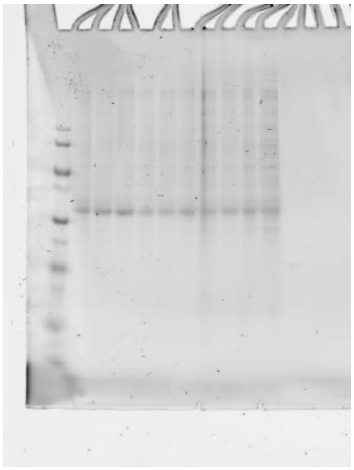

Figure 5. SDS-PAGE gel with SYPRO red staining

The images were quantified using imageJ gel band quantification toolkit. The Halo-CB2-eGFP band was quantified by taking the area under the curve, normalising this using the total protein amount quantified using the SYPRO red staining and averaging this value over the duplicates for each condition. The fluorescence was then normalised by setting the 0 hour to 100%. The normalized data were then plotted in Prism. Error bars indicate standard error of the mean (SEM).

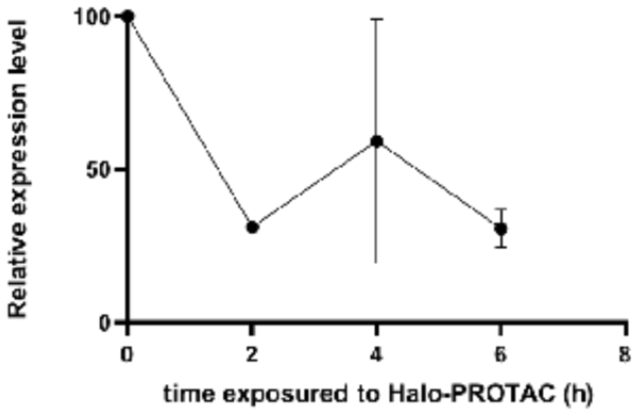

Figure 6. The relative amount of Halo-CB2-eGFP after 0, 2, 4 or 6 hours of treatment with Halo-PROTAC-E. Error bars are in SEM.

This graph shows a downward trend, which means that the HALO-CB2-eGFP is degraded over time. The outline at 4 hours is probably caused by:

1. The absolute expression of a (recombinant) protein depends on many factors including the transfection efficiency. This introduces a variability in the outcome.
2. Because there are only two experimental sample data, it is prone to cause error

Localisation of Halo-CB2-eGFP

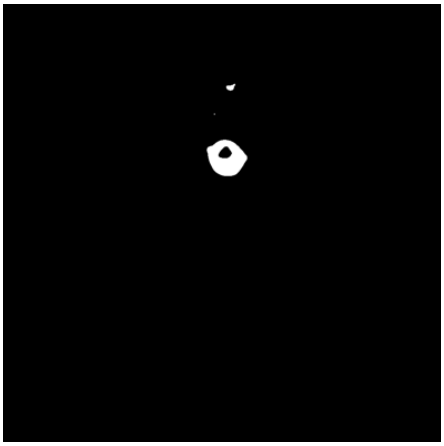

Figure 7. A representative confocal image

Mander's coefficient is used to quantitatively determine the correlation of co-localization in the cell, so that two molecules can be determined whether they interact with the same complexes in the cell.

In order to know whether the degradation happens on the ER membrane or the cell membrane, I quantified the overlap of the eGFP fluorescence with the DIL stain (plasma membrane) and ER cytopainter (ER). Through Mander's overlap coefficient, the extent of colocalization can be quantified. The results are depicted in figure 8.

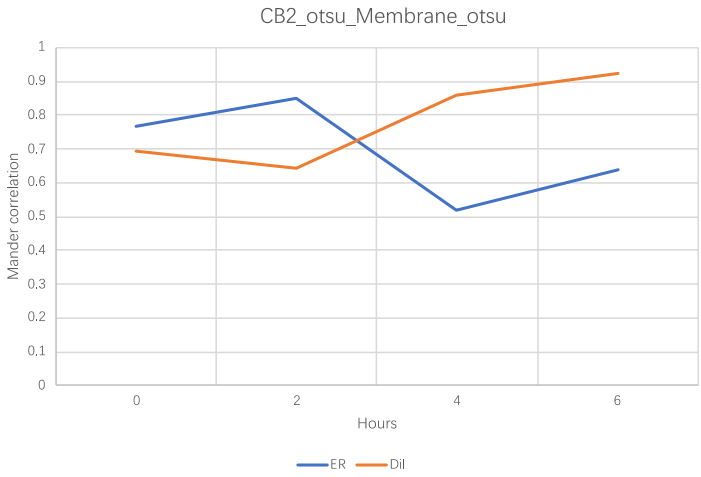

Figure 8. Mander's correlation for ER and Dil stains at 0,2,4 and 6 hours

From the figure 8, the overall trend of ER is downward, while the trend of Dil is upward, which means the degradation happens on ER, since it is a degradation process, the correlation should generally decrease through time.

Another way to look at the degradation is to quantify the amount of receptor. In this case is to measure the mean intensity of CB2 correlates with membrane.

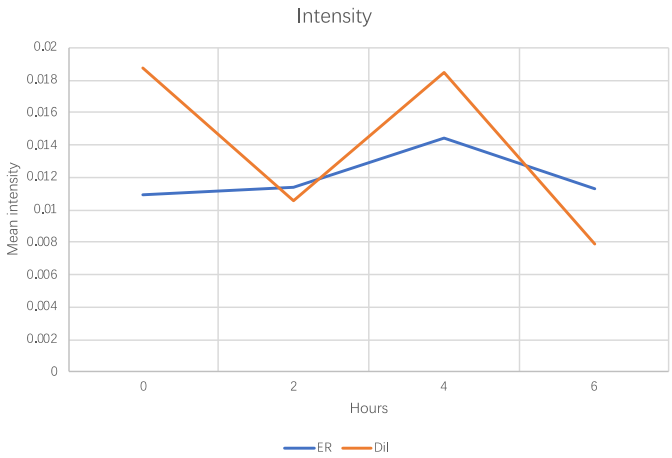

Figure 9. mean intensity of CB2 correlates with membrane

From figure 9, the trend for ER membrane is rather flat, while the trend for Dil is decreasing. However, it is contrary to the figure 2 that the trend for Dil stain is increasing. The reason for the difference remains for further studies.

Conclusion

By using HaloPROTAC-E, we found that Halo-CB2-eGFP can be degraded after Halo-PROTAC-E binds to N-terminal linked HaloTag and we found that the degradation happens in the ER. The experiments need to be repeated in order to reach statistical significance.

PROTAC can use the natural protein cleaning system in the body to reduce protein level rather than inhibit protein function. Therefore, the study of HaloPROTAC and its reaction mechanism will lay the foundation for the development of related drugs in the future. Although the reaction mechanism for the degradation of extracellular cell remains unknown, it will be solved in the near future.

Learning outcomes

These weeks' experience gave me a practical knowledge of how things are done and practiced in the real lab. The lab experience provided me the exposure of lab training. It helped me to learn how to manipulate various experimental machines, details of cloning protocols and working in tissue culture. Using several software packages to analyze data allowed me to better understand the theoretical basis of the experiment and biology knowledge. Biology, unlike chemistry, it is the study of living cells. Each cell even under same conditions may express differently, therefore it requires patience of redoing experiments repeatedly. But that makes it interesting because you would never know what would happen next.

References

1. Benredjem, B., Dallaire, P. and Pineyro, G., 2017. Analyzing biased responses of GPCR ligands. Current Opinion in Pharmacology, 32, pp.71-76.
2. Buckley, D.L., Raina, K., Darricarrere, N., Hines, J., Gustafson, J.L., Smith, I.E., Miah, A.H., Harling, J.D. and Crews, C.M., 2015. HaloPROTACS: use of small molecule PROTACs to induce degradation of HaloTag fusion proteins. ACS chemical biology, 10(8), pp.1831-1837.