# Investigating the signaling mechanism of GPER via BRET

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## Abstract

Female sex and history of prior pregnancies are associated with favorable melanoma outcomes, and past research from the Ridky Lab has shown that this melanoma protective effect likely results from estrogen signaling through the G protein-coupled estrogen receptor (GPER) on melanocytes. While the signaling mechanism is thought to be through cyclic adenosine monophosphate (cAMP) activation of protein kinase A (PKA), as GPER is thought to be a $G_s$-coupled receptor, there is still some uncertainty regarding the alpha subunit of GPER's associated G protein. This project seeks to answer this question through the cloning of GPER into a PRRL vector and the use of bioluminescence resonance energy transfer (BRET) to confirm or reject the idea that GPER is $G_s$-coupled. Deeper knowledge of the nature of this receptor could lead to better understanding of this melanoma protective effect and, consequently, optimized treatment options for melanoma and other cancers.

## Background

### GPER Signaling

GPER is thought to be a $G_s$-coupled G protein-coupled receptor (GPCR), which implies it stimulates cAMP production and consequently activates PKA.

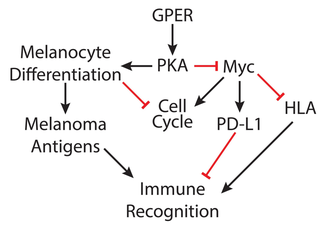



**Figure 1.**[1] Proposed mechanisms for protective effect of GPER.

### GPCRs and BRET

GPCRs are the largest family of Bproteins targeted by approved drugs. These receptors are coupled to G proteins: heterotrimeric proteins composed of alpha, beta, and gamma subunits. Activation of a GPCR by its respective ligand causes the alpha subunit to dissociate from the beta and gamma subunits, and the alpha subunit plays the main role in further signaling through interaction with other proteins. A GPCR can have 1 of 16 alpha subunits, and different subunits are associated with different signaling mechanisms. BRET is a novel technique to determine a GPCR's endogenous alpha subunit. It relies on the transfer of energy from an energy donor (RLuc) attached to the alpha subunit to an energy acceptor (GFP) attached to the beta subunit in order to determine whether the alpha subunit has dissociated. Spectroscopy is used at wavelengths of 400 nm and 510 nm to quantify light emitted by Rluc and GFP respectively, the ratio of the two telling of alphas subunit dissociation and, indirectly, GPCR activation.

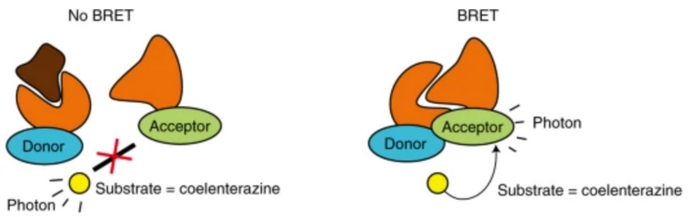



**Figure 2.**[2] Graphic illustrating biological interactions behind BRET.

## Methods

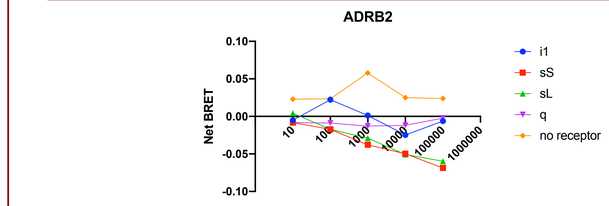
### Cloning GPER (WT and P16L) into PRRL

- Purpose: To create WT and P16L GPER plasmids in a PRRL vector for future use in the lab.
- Day 1: PCR
  - Add 10 uL 5X Q5 buffer, 1 uL 10 mM dNTP mix, 2.5 uL GC enhancer, 1 uL Q5 DNA polymerase, and nuclease-free water up to 50 uL in a PCR tube for each construct.
  - Run on PCR: 30 seconds 98C; 40 cycles 8 seconds 98C, 20 seconds 62C, 25 seconds 72C, and 2 minutes 72C; and finally hold 4C overnight.
- Day 2: PCR Clean Up, Digestion, Gel Extraction, and DNA Quantification
  - Follow PCR Clean Up Kit.
  - Ad 1 ug DNA, 5 uL of 10X CutSmart Buffer, 1 uL SalI-HF, 1 uL AgeI-HF, and nuclease-free H2O up to 50 uL in an Eppendorf tube for each construct. Make one construct for undigested DNA (negative control), one digested with only AgeI, one digested with only SalI, and one digested with both enzymes; all these constructs use PRRL as the DNA. Make one construct with WT DNA digested by both enzymes and one with P16L DNA digested by both enzymes.
  - Incubate at 37C for about 1 hour.
  - Run each of the six constructs on a 0.7% agarose gel at 140V with 10 uL of 6X loading dye, with a DNA ladder flanking each side, until the bands reach halfway.
  - Cut out the PRRL backbone (the brightest band in the fourth construct) and each of the samples (the last two constructs).
  - Follow the gel extraction kit to extract the DNA.
  - Quantify the DNA using the Nanodrop.
- Day 3: Ligation
  - Add 25 ng insert, 50 ng vector, 1 uL 10X buffer, 1 uL T4 ligase, and fill to 10 uL with nuclease-free H2O.
  - Incubate at 16C overnight.
- Day 4: Transformation
  - Follow the steps of a Stbl3 transformation. Plate 2 plates for each, with 1 being 50 uL of the solution and 1 being 50 uL of a 1:10 dilution (6 total: no insert, WT, and P16L).
- Day 5: Single Colony Selection
  - Continue to follow Stbl3 transformation protocol, moving the plates to 4C for 5 hours after parafilming them and then inoculating 2 vials per plate with a single colony each (only 1 plate of each condition used, being the better one for isolation of a single colony).
  - Put vials in shaker overnight at 225 rpm with lids allowing air in.
- Day 6: Miniprep
  - Follow miniprep kit and quantify DNA concentration.
  - Send plasmids for sequencing to confirm identity

### BRET

- Purpose: To analyze the endogenous alpha subunit of GPCR's.
- Day 1
  - Plate 700K HEK293T cells/well in a 6-well plate in DMEM 5% FBS. 1 well is needed for each condition (alpha subunit/receptor combo).
  - Incubate 6-8 hours in 37C.
  - Warm TransIT-2020 to room temperature and vortex gently.
  - For each condition, place 40 uL Opti-MEM I Reduced-Serum Media in a sterile tube.
  - Add plasmid DNA at a 1:1:1:1 DNA ratio of receptor:GA-RLuc8:GB:GG-GFP2 (100 ng per construct).
  - Pipet gently to mix completely.
  - Add 1.3 uL TransIT-2020 per tube.
  - Pipet gently to mix completely.
  - Incubate at room temperature for 15-30 minutes to allow complexes to form.
  - Add to appropriate well in the 6-well plate.
  - Incubate overnight at 37C.
- Day 2
  - Trypsinize each well with 500 uL of trypsin and 1 mL DMEM 5% FBS to neutralize.
  - Count the cells in each well.
  - Plate 30K cells/well in a 96-well plate coated with poly-D-lysine.
  - Incubate overnight at 37C.
- Day 3
  - Add Perkin Elmer white backing to back of 96-well plate.
  - Aspirate growth media and replace with 60 uL of assay buffer (1X Hank's balanced salt solution/HBSS + 20 mM HEPES, pH 7.4 without phenol red).
  - Make coelenterazine and drug solutions.
    - Coelenterazine: Get 20 uL aliquot of 5 mM coelenterazine from -180C and fill to 2 mL with HBSS.
  - Add 10 uL of 50 uM coelenterazine to each well and wait 5 minutes with foil over plate (light-sensitive).
  - Add 30 uL of drug solutions to wells and wait 5 minutes again with the foil.
  - Read the plate on the plate reader serially 6 times, making sure to adjust to BRET2 settings.

## Data and Results

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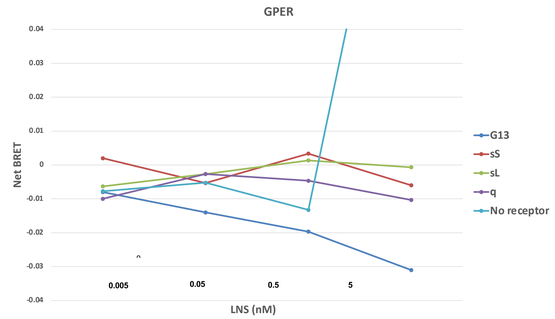
|---|---|---|---|---|---|---|---|
| i1 | -0.011 | i2 | -0.023333 | i3 | 0.005333 | oA | 0.004667 |
| oB | -0.000333 | Z | -0.003333 | Gus | -0.01 | sS | -0.018667 |
| sL | -0.032333 | Q | -0.023 | 11 | -0.038 | 15 | -0.022667 |
| 12 | -0.038 | 13 | -0.106333 | no receptor | -0.0415 | | |




- The first graph illustrates the results of a BRET assay with ADRB2 and isoproterenol (control)
- The table illustrates the net BRET results of a BRET assay testing all alpha subunits with GPER and 100 nm LNS
- The second graph illustrates the results of a BRET assay with GPER and LNS

## Discussion & Future Directions

The results show that GPER may potentially be a $G_{13}$-coupled receptor. The BRET performed transfecting the cells with all alpha subunits available with the TRUPATH technology, the respective beta and gamma subunits, and GPER highlighted $G_{13}$ as potentially being the endogenous alpha subunit. The fact that the BRET performed with differing doses of LNS and transfecting the cells with only GPER and the possible endogenous subunits along with their respective beta and gamma subunits deduced from the first BRET gave the cleanest dose curve instead of random scattering of net BRET values tells that $G_{13}$ may likely be the endogenous alpha subunit of GPER. In the future, further assays will be performed to confirm GPER is $G_{13}$-coupled and elucidate the precise signaling mechanisms of this receptor. With a deeper understanding of the signaling of GPER, there will consequently be a deeper understanding of GPER's melanoma protective effect. This will likely allow for improved treatments of melanoma and other somatic cancers, as combinatorial therapies will likely be able to be created with molecules that make use of GPER's protective effect, anti-PD-1 therapy (the standard of care for melanoma), etc. If it is found GPER is not $G_{13}$-coupled, further research will be needed to determine its endogenous alpha subunit and to use this information to create therapies making use of its protective effect.

## Acknowledgements and References

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[1] Natale, Christopher A, et al. "Author Response: Activation of G Protein-Coupled Estrogen Receptor Signaling Inhibits Melanoma and Improves Response to Immune Checkpoint Blockade." 2017, doi:10.7554/elife.31770.017.

[2] Kobayashi, Hiroyuki, et al. "Bioluminescence Resonance Energy Transfer–Based Imaging of Protein–Protein Interactions in Living Cells." *Nature Protocols*, vol. 14, no. 4, 2019, pp. 1084–1107., doi:10.1038/s41596-019-0129-7.