

Eicosanoids: Key Actor Proteins in Autoimmune Diseases and Laminopathies

Characterizing a New Nuclear Envelope Protein and its Role in Asthma and Allergies

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I. SPECIFIC AIMS

FLAPv2 is a recently discovered isoform of the nuclear envelope integral membrane protein arachidonate 5-lipoxygenase activating protein (FLAP). FLAP collects the substrate arachidonic acid (AA) and biosynthetic enzymes, arachidonate 5-lipoxygenase (5- LO), leukotriene A4 hydrolase, leukotriene C4 synthase, together into clusters to generate eicosanoids, in particular leukotrienes (LTs). LTs play a major role in autoimmune and inflammatory diseases, ranging from asthma to cardiovascular disease.

We hypothesize that FLAPv2 is also involved in leukotriene synthesis and it is a possible target for drug therapy. We will test this hypothesis by isolating and purifying proteins from the nuclear envelope of myeloid cells. Our specific aims are:

1. To characterize the recently discovered isoform of FLAP, FLAPv2, and to determine if both isoforms, FLAPv1 and FLAPv2, are in the same cellular space (nuclear envelope) and interact with each other to produce leukotrienes.
2. To refine our understanding of the interactions of FLAPv2, with FLAPv1, AA, and 5-LO, which may reveal novel pathways of leukotriene synthesis and provide insights for potential drug therapy targets.

II. BACKGROUND AND SIGNIFICANCE

Autoimmune diseases such as asthma, rheumatoid arthritis, type 1 diabetes, and multiple sclerosis occur when your immune system mistakes your own tissues as foreign and attacks them. Autoimmune diseases in part involve an overproduction of cytokines and chemokines which result in tissue inflammation. Similarly, in allergies, your immune system flags harmless environmental allergens or substances as dangerous and goes on an “attack mode,” which also results in tissue inflammation.

Myeloid cells and specifically myeloid leukocytes are key actors during an innate immune response, in part through the production of the pro-inflammatory bioactive signaling molecules called leukotrienes (LTs). LTs are lipid-signaling molecules derived from arachidonic acid (AA) that initiate and amplify inflammation. When your body’s antibodies (type IgE) encounter an allergen, they bind to receptors on the surface of your myeloid cells. The receptors link together and become active, signaling the synthesis and release of LTs from that myeloid cell(*quote*).

The initial steps in the synthesis of LTs are the translocation of 5-lipoxygenase (5-LO) to the nuclear envelope and its subsequent association with its scaffold protein 5-lipoxygenase-activating protein (FLAP). FLAP collects the substrate AA and biosynthetic enzymes, 5- LO, leukotriene A4 hydrolase, leukotriene C4 synthase, together into clusters to generate LTs. One of these LTs, LTB4, causes vasodilation and mucosal secretion in airways, leading to allergic rhinitis. Another leukotriene, LTC4, is implicated in bronchoconstriction, leading to asthma (*quote*).

Although there is a gap in our understanding of the exact process in which the organization of 5-LO and FLAP on the nuclear envelope regulate LT synthesis, we understand that FLAP is essential for the initiation of LT biosynthesis. FLAP is considered to function as a scaffold protein that brings 5-LO, which in resting cells resides in cytosol and the nucleoplasm, in proximity with AA (*quote*).

Recently, we discovered that FLAP has an isoform, FLAPv2, which has a unique region of 57 amino acids that the original variant does not have. The original variant, which we will refer to as FLAPv1 moving forward, has a molecular weight of 18,157 Da, while FLAPv2 has a molecular weight of 24,207 Da, due to the extra region (*see appendix 1*). What we know about FLAPv1 and FLAPv2 is that they are both present in mammalian cells, as they were detected through Western Blot and microscopy performed on RBL and RAW cells.

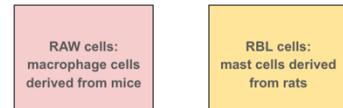
We want to determine if FLAPv1 and FLAPv2 interact, and if they are both in the same cellular space (nuclear envelope). We also want to determine if FLAPv2 is required for LTs biosynthesis. In addition, we want to understand if FLAPv2 also interacts with the other components that required for LT biosynthesis, 5-LO and AA, and through what pathways. Characterizing FLAPv2 and refining our understanding of the role of FLAP proteins in LT production has the potential of discovering new roles or novel FLAP-binding proteins involved in autoimmune disease and tissue inflammation, which can be potential drug therapy targets.

III. PRELIMINARY STUDIES

We used RBL-2H3 (mast cell-like cell line) and RAW.264.7 (macrophage cell-like line) systems to isolate and characterize the nuclear envelope. We cultured RAW, RBL, and transvected RBL with FLAP lentivirus GFP (green fluorescent). RAW and RBL cells contain endogenous FLAPv1 and FLAPv2. Lenti-RBL cells are expected to have higher FLAP expression. In addition, we used the following tools:

- FLAPv1 and FLAPv2 affinity purified antibodies (the FLAPv2 antibody was created using the protein's unique amino acid region)
- FLAPv1 with Histidine tag expressed in E.coli culture
- FLAPv1 without Histidine tag expressed in E.coli culture
- FLAPv2 with Histidine tag expressed in E.coli culture
- FLAPv2 without Histidine tag expressed in E.coli culture

Maintaining mammalian cells that contain FLAPv1 and FLAPv2



~Both well suited for the study of allergies~

Bacteria (E.coli) with FLAPv1 and FLAPv2 plasmids



Experiments/Methods

Figure 1 (citation)

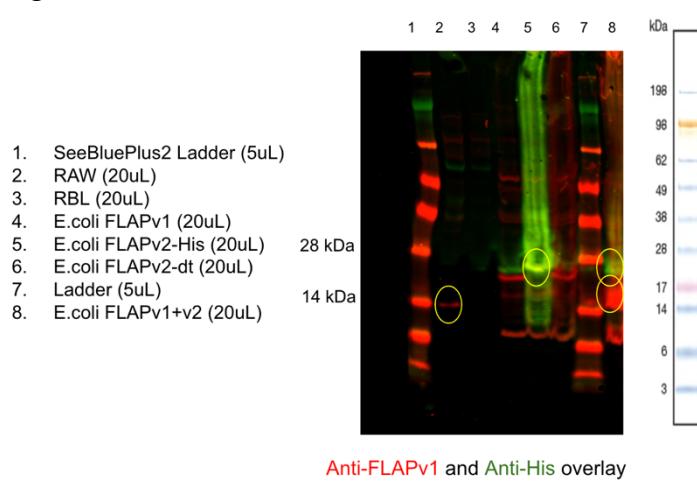
1. Western Blot

With the purpose to identify if FLAPv1 and FLAPv2 coexist and if they are cross-linked, we performed Western Blot in RAW cells, RBL cells, Lenti RBL cells, and bacterial cells (E.coli) which were cultured to express FLAPv1 and FLAPv2 with and without Histidine tags.

Western blotting uses antibodies to identify individual proteins within a cell or tissue lysate. Antibodies bind to highly specific sequences of amino acids, which vary from protein to protein. First, proteins are separated from each other based on their size by polyacrylamide gel as a support medium and sodium dodecyl sulfate, also known as SDS-PAGE gel electrophoresis. Next, the proteins are transferred from the gel to a membrane by application of an electrical current. The membrane can then be processed with primary antibodies specific for target proteins of interest. Next, secondary antibodies are added for detection of the antibody/protein complex.

Using our sample cells, we prepared cell lysates and we boiled them for 5 minutes. We inserted the gel into the SDS-PAGE tank and filled the chambers of the tank with Bolt MES SDS running buffer. Next we pipetted in the gel 5ul of the protein ladder followed by our sample lysates. For the ladder we used SeeBluePlus2 Prestained Standard. Our lysates were inserted in the following concentrations and sequence, 20ul RAW cell lysate, 20ul RBL cell lysate, 20ul E.coli FLAPv1 lysate, 20ul E.coli FLAPv2+Histidine lysate, 20ul E.coli FLAPv2, 20ul E.coli with co-expressed FLAPv1 & FLAPv2 lysate. After electrophoresis was completed, the proteins were transferred from the gel onto a membrane for antibody staining and detection. Transfer was performed by passing a current across the gel to the membrane. After the transfer, we stained the membrane with Ponceau red to ensure that protein transfer was successful. We performed immunoblot with 6x-His Tag Monoclonal Antibody (Mouse anti-6x-His), Goat anti-FLAPv1, and Rabbit anti-FLAPv2. We incubated membrane in blocking solution overnight at 4°C with constant rocking. Next, we applied the following secondary antibodies, anti-Mouse His, donkey anti-Goat, and donkey anti-Rabbit, and we added a solution of PBST and blocking buffer (half and half). We incubated protected from light for 10 minutes and then we scanned the membrane.

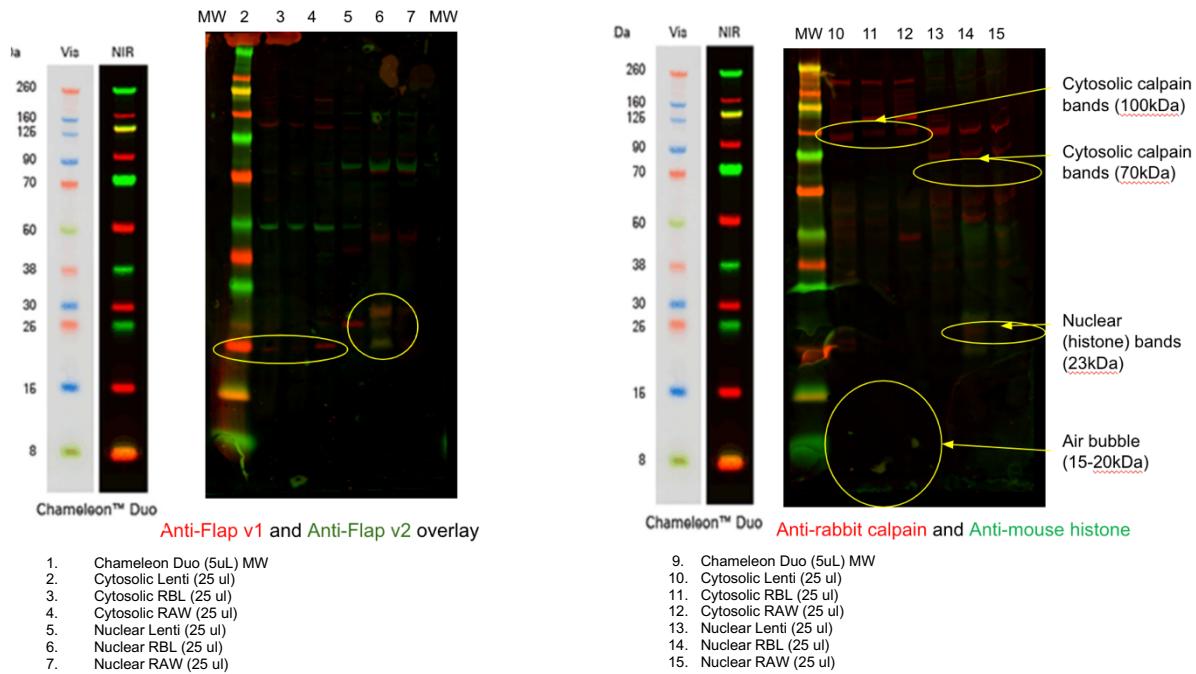
The results showed us that both proteins were expressed in E.coli and were identified by anti-FLAPv1 and anti-His. We determined that FLAPv1 coexists with FLAPv2, but we could not infer that they were crosslinked, because the bands were separated. We also detected FLAPv1 in RAW cells, but not FLAPv2, most likely because FLAPv2 was only detected by the histidine tag, which was not present in the mammalian cells.



Consequently, we performed another Western Blot, following the same protocol, but this time we used lysates from our mammalian cells that we previously fractionized into nuclear and cytoplasmic. The objective was to identify if FLAPv1 and/or FLAPv2 exists in the nucleus, cytoplasm, or both.

For this experiment we used 5ul of a Chameleon Duo ladder, and we loaded the gel in the following sequence, Chameleon Duo (5uL), Cytoplasmic Lenti lysate (25ul), Cytoplasmic RBL lysate (25ul), Cytoplasmic RAW lysate (25ul), Nuclear Lenti lysate (25ul), Nuclear RBL lysate (25ul), Nuclear RAW lysate (25ul). We also loaded a second ladder followed by Cytoplasmic Lenti lysate (25ul), Cytoplasmic RBL lysate (25ul), Cytoplasmic RAW lysate (25ul), Nuclear Lenti lysate (25 ul), Nuclear RBL lysate (25ul), and Nuclear RAW lysate (25ul). The goal was to use the second half of the membrane as a control to ensure that the fractionization of the nuclei and cytoplasm was successful. Therefore, after transferring the gel to the membrane, we split the membrane into two parts. On the first part we applied the antibodies anti-FLAPv1 goat and anti-FLAPv2 rabbit, and on the second part we applied calpain from rabbit (an antibody that will bind cytosolic proteins) and histone from mouse (an antibody that will bind nuclear proteins). For secondary antibodies, we used anti-Goat 680nm and anti-Rabbit 800nm for the first part of the membrane, and anti-Rabbit 680nm and anti-Mouse 800nm in the second half.

The results showed us some FLAPv1 in RAW cells at the cytoplasmic fraction but not in the nuclear fraction. In RBL cells, we were able to detect both FLAPv1 and FLAPv2 in the nuclear fraction. This finding is promising as it is consistent with our first specific aim that both FLAPv1 and FLAPv2 are located in the nuclear envelope, therefore, we will need to evaluate this further by repeating the experiment. The control membrane appeared as expected with bands of calpain in the cytoplasmic cells and histidine bands in the nuclear cells.



2. Electron Microscopy

To identify the localization of endogenous FLAPv1 and FLAPv2 in the cell, we performed immunogold-electron microscopy (EM), which provides a high-resolution imaging inside the cell. Similar to immunoblotting, in EM we are labeling the protein of interest, but instead of a fluorophore attached on the secondary antibody, a gold particle is attached to the protein's antibody. Gold has a high electron density, so it scatters more electrons that appear as dark spots on an image.

We fixed RAW and RBL cells with glutaraldehyde and we immunogold labeled the primary antibodies for FLAPv1 and FLAPv2. For both RBL and RAW cells, the images showed us that both isoforms protein were in both inner and outer space of the nuclear envelope, with few spots closer to the membrane. We also observed FLAP on the mitochondrial membrane (*see appendix 2 for EM images*).

We were not able to determine if FLAPv1 and FLAPv2 concentrate on the nuclear envelope, therefore we decided to repeat the experiment with activated (stimulate the leukotriene cascade to produce more FLAP). We induced RAW and RBL cells with lipopolysaccharide (LPS), and we decided to test if they are activated with enzyme-linked immunosorbent assay (ELISA). This experiment is described below. We sent the fixated cell for a second EM and we are waiting for the results.

3. ELISA (Enzyme-Linked Immunosorbent Assay)

As described above, the purpose of the ELISA Functional Assay is to determine if our RBL and RAW cell lines were successfully activated. ELISA is a plate-based assay technique in which a substance is detected/quantified using an enzyme reaction with its substrate. The enzyme's activity is measured using its substrate that changes color when modified by the enzyme. Light absorption of the product formed after the substrate addition is measured and converted to numeric values (citate).

To decide if our cell lines were stimulated after the LPS treatment, we used, for RAW cells, an ELISA that measures PGE₂, a prostaglandin produced by RAW cells (macrophage cell-like) and for RBL cells, an ELISA that measures LTC₄, a leukotriene that is produced by RBL (mast cell-like). Both eicosanoids PGE₂ and LTC₄ are over expressed during tissue inflammation.

Our ELISA results demonstrated high concentrations of both eicosanoids (*see appendix 3 for graphs and tables*), which indicated that our cells were successfully activated. Moving forward, we plan using the activated cells to detect FLAPv1 and FLAPv2 using their antibodies, through immunoblotting, electron microscopy, and other protein detection techniques.

4. dSTORM (Direct Stochastic Optical Reconstruction Microscopy)

dSTORM is a super-resolution fluorescence imaging technique, which allows the observation of cellular processes with molecular specificity through fluorophores, which fluorophores, which can be converted to an off state using specific excitation parameters. We used this method to detect FLAPv1 and FLAPv2 in previously treated to isolate nuclei in mammalian Lenti-RBL and RAW cells lines. We anti-FLAPv1 an antibody that detects FLAPv1 and FLAPv2, anti-FLAPv2, an antibody that detects FLAPv2 only due to its unique sequence, and a 6xHis tag connected to FLAPv1 only. We expected to detect both protein isoforms in the nuclear envelope and ideally concentrated around the nuclear membrane.

Our results demonstrated that in Lenti-RBL cell nucleus, both FLAPv1 and FLAPv2 were in the nuclear envelope, but they were dispersed and it was not clear if they and there was concentrated around the nuclear membrane. There was, however, an area in which FLAPv1 seemed to be over-expressed, which was expected from the transfected Lenti-RBL cells. An explanation could be that this particular over-expressed are is the nuclear membrane folding into the Golgi complex, where FLAP molecules are produced.

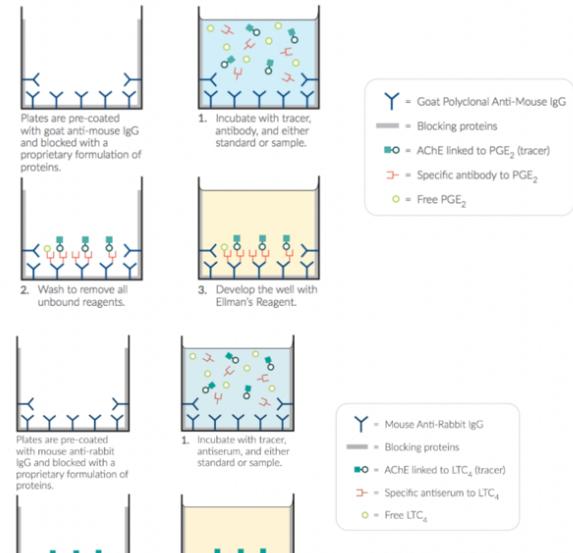
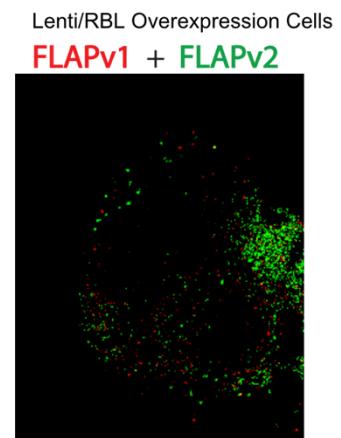
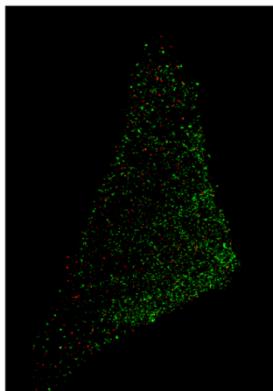


Figure 2:
PGE₂ ELISA
& LTC₄ ELISA
illustration



Lenti/RBL Overexpression Cells

FLAPv2 + 6xHis(FLAPv1)



As we mentioned above, the anti-FLAPv1 antibody recognizes both isoforms, therefore we could not be certain if we were viewing FLAPv1 exclusively in our image. Therefore, in a second image that we again processed on Lenti-RBL cells, we utilized an antibody against the 6xHis tag found exclusively on FLAPv1, in order to be able to discriminate FLAPv1 exclusively. The results were similar to the previous image, both FLAPv1 and FLAPv2 appear, and FLAPv1 is over-expressed compared to FLAPv2.

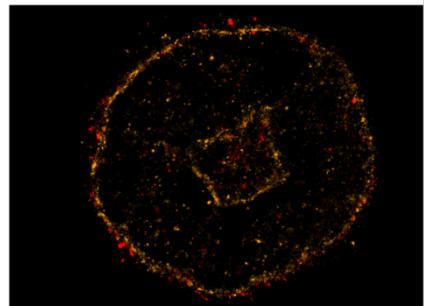
Finally, we imaged the RAW cell line nucleus, which revealed us both proteins being present in the nuclear envelope, but again both FLAPv1 and FLAPv2 are dispersed and there

is no clear indication that they are concentrated around the nuclear membrane.

To get more clear results of the localization of the isoforms, we intend to repeat the experiment using preincubated FLAPv1-FLAPv2 overlay with purified forms of FLAPv1 and FLAPv2, to ensure that the anti-FLAPv1 will not interact with both isoforms. Another idea is to perform dSTORM on activated RAW and RBL cells. We also intend to perform a cell fractionation and analyze separate nuclear fractions.

RAW Cells

FLAPv1 + FLAPv2



IV. RESEARCH DESIGN AND METHODS

The goal of this proposal is to characterize FLAPv2 and determine its role in eicosanoid production, which would potentially provide us with novel information on leukotriene synthesis, thus new drug therapy targets. To achieve our first specific aim, we intend to carry out a more detailed examination of the interaction between FLAPv1 and FLAPv2, and ascertain the cross-linkage between the two protein isoforms in relation to eicosanoid production. To achieve our second specific aim, we intend to examine the interactions of FLAPv2 with 5-LO and AA, by using protein-protein interaction techniques.

Experiments to be performed

1. Perform additional immunoblotting on LPS activated RBL-2H3 (mast cell-like cell line) and RAW.264.7 (macrophage cell-like line) to detect whether the two isomers, FLAPv1 and FLAPv2, are cross-linked.
2. Perform a site directed mutagenesis with the purpose to remove the histidine tag from pIRESbleo3-FLAPv2 so that FLAPv1 can be uniquely identified by immunoblot with its histidine tag. Currently, we have an antibody that recognizes both FLAPv1 and FLAPv2, and an antibody that recognizes FLAPv2 only. We would like to be able to identify if the two isoforms are cross-linked. If we remove the histidine tag from FLAPv2, then we will

perform immunoprecipitation and have histidine bind to the magnetic beads. We would then purify and see if we find both isoforms, which would manifest cross-linkage between FLAPv1 and FLAPv2, since only FLAPv1 would have the His-tag.

3. Perform a cell fractionation and immunoprecipitation: separate cytosol, mitochondria, nuclear fractions and analyze these fractions by western blot to determine if the proteins are at the nuclear membrane or in other organelles.
4. Mass spectrometry (+/- cross-linking) to determine interactions and what proteins bind to FLAPv2 (specific aim 2)
5. Identification of the FLAPv2 binding proteins using affinity purification with either immobilized FLAPv2 or immobilized anti-FLAPv2 antibodies. After purification, the partial N-terminal amino acid sequence of the binding partners will be determined and then used to identify these proteins.
6. Stimulate cells and then crosslink their proteins using DMP (dimethyl pimelimidate-2 HCl) and identify complexes with SDS-PAGE and a proximity ligation assay.

Notes:

Use existing experimental approaches in your proposal It is not cheating: 95% of research is based on recycled experimental approaches They are validated approaches: strengthen your proposal Experimental approaches should be: • Founded on approaches known to work • Applicable to your own project • Straightforward

What do you include in the description of each experiment? Correlation with the “What 4X” design thinking approach What is: background, preliminary data (Exploring the current reality) What if: hypotheses (Envisioning alternative futures) What wows: Convincing readers of your proposals’ feasibility & quality (Getting users to help making tough choices) What works: Proposing sound and validated experimental approaches (making it work in-market and as a business)

For each experiment: • Brief overview of the experimental approach • Likely outcome • Controls • Possible limitations and pitfalls • Logical link to the next experiment Types of experiments for 1 specific aim: • Sequential experiments • Convergent experiments • Complementary experiments)

APPENDIX

1. FLAP Variants

Region Unique to variant 2

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ATGCTCACATTAATCACGATGCTCCCTGGCATACACAGAAGACTCTGAAAACCTTCTGAA
M L T F N H D A P W H T Q K T L K T S E
TTTGGGAAATCCTTGGCACCTTGGGCACATTGGAACATAAGCCATCAGTGCTGGCA
F G K S F G T L G H I G N I S H Q C W A
GGTTGTGCAGCTGGAGGCAGAGCAGTCCTCTGGGAGCCTGAAGCAAACATGGATCAA
G C A A G G R A V L S G E P E A N M D Q
GAAACTGTAGGCAATGTTGCTCTGTTGCCATCGTCACCCTCATCAGCGTGGTCCAGAAT
E T V G N V V L L A I V T L I S V V Q N
GGATTCTTGCCCATAAAAGTGGAGCACGAAAGCAGGACCCAGAACATGGGAGGAGCTTCAG
G F F A H K V E H E S R T Q N G R S F Q
AGGACCGGAACACTTGCCCTTGAGCGGGCTACACTGCCAACAGAACTGTGTAGATGCG
R T G T L A F E R V Y T A N Q N C V D A
TACCCCACCTTCCTCGCTGTGCTCTGGTCTGCGGGGCTACTTGCAGCCAAGTTCCCTGCT
Y P T F L A V L W S A G L L C S Q V P A
GCGTTTGCTGGACTGATGTACTTGTGAGGCAAAAGTACTTGTGCGTTACCTAGGA
A F A G L M Y L F V R Q K Y F V G Y L G
GAGAGAACGCAGAGCACCCCTGGCTACATATTGGAAACGCATCATACTCTTCCTGTT
E R T Q S T P G Y I F G K R I I L F L F
CTCATGTCCCGTTGCTGGCATATTCAACTATTACCTCATCTTCTTTCGGAAGTGACTTT
L M S V A G I F N Y Y L I F F F G S D F
GAAAACATACATAAGACGATCTCCACCACATCTCCCTCTACTTCTCATTCCCTAA
E N Y I K T I S T T I S P L L L I P -

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Sequence alignment (underlined sequence: used to create the anti-V2 antibody)

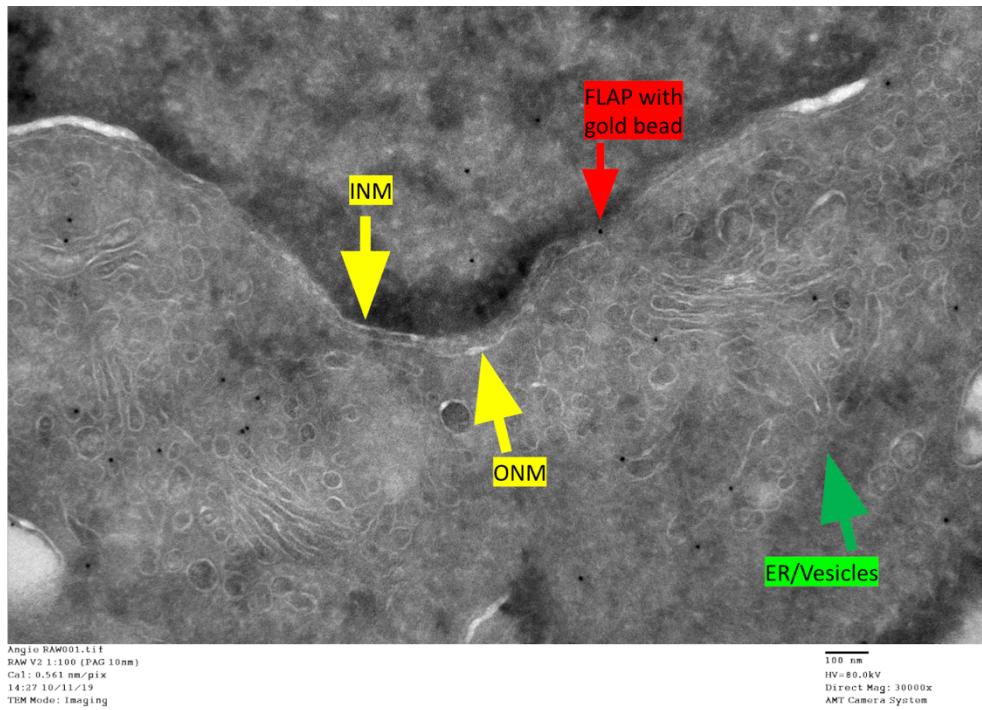
FLAP1	1	-----
FLAP2	1	<u>MLTFNHDAPW HTQKTLKTSE FGKSFGLGH IGNISHQCWA GCAAGGRAVL</u>
FLAP1	1	-----MDQ ETVGNVVLLA IVTLISVVQN GFFAHKVEHE SRTQNGRSFQ
FLAP2	51	<u>SGEPEAN</u> MDQ ETVGNVVLLA IVTLISVVQN GFFAHKVEHE SRTQNGRSFQ
FLAP1	44	RTGTLAFERV YTANQNCVDA YPTFLAVLWS AGLLCSQVPA AFAGLMLFV
FLAP2	101	RTGTLAFERV YTANQNCVDA YPTFLAVLWS AGLLCSQVPA AFAGLMLFV
FLAP1	94	RQKYFVGYLG ERTQSTPGYI FGKRIILFLF LMSVAGIFNY YLIFFFGSDF
FLAP2	151	RQKYFVGYLG ERTQSTPGYI FGKRIILFLF LMSVAGIFNY YLIFFFGSDF
FLAP1	144	ENYIKTISTT ISPLLLIP
FLAP2	201	ENYIKTISTT ISPLLLIP

Molecular weight:

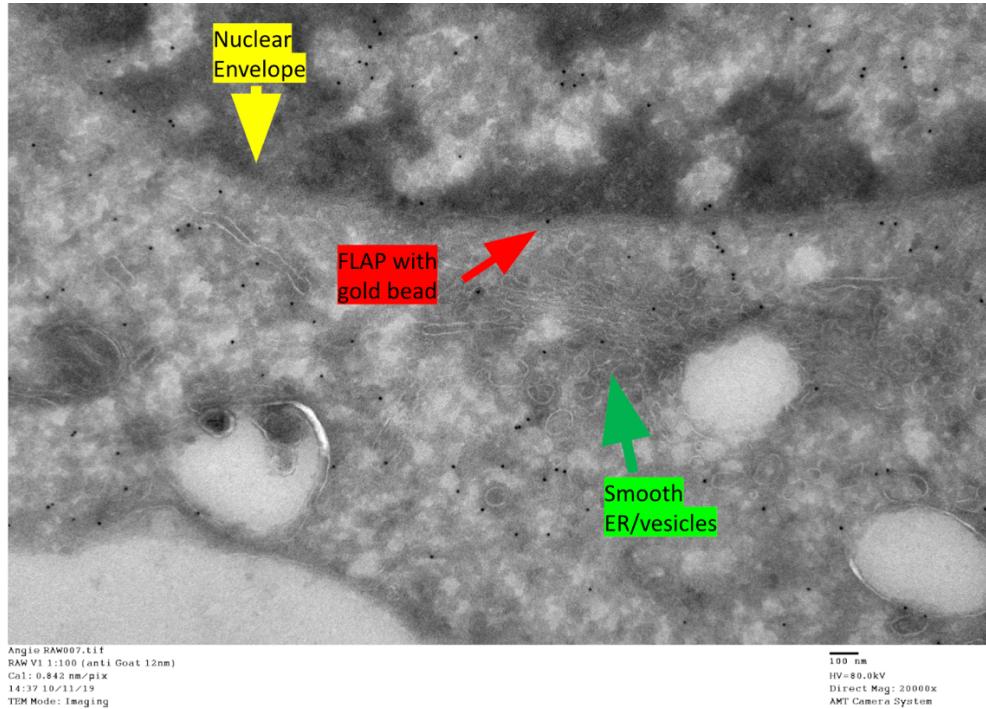
FLAP_V1: 18157 Da
FLAP_V2: 24207 Da

2. Electron Microscopy Images

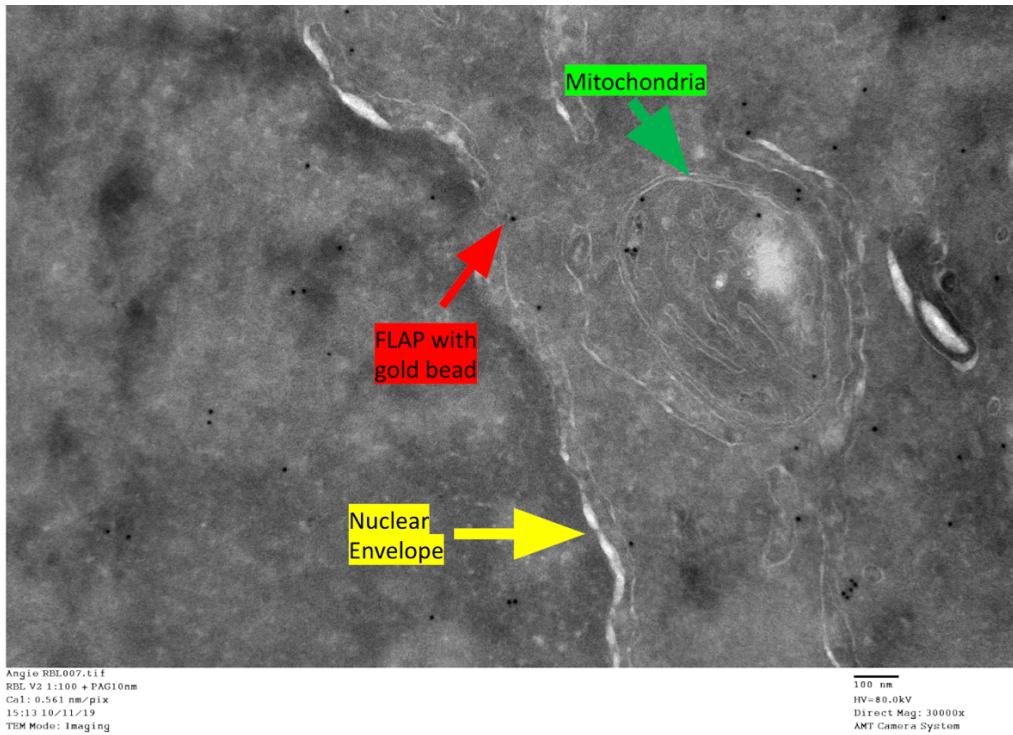
FLAPv2 RAW



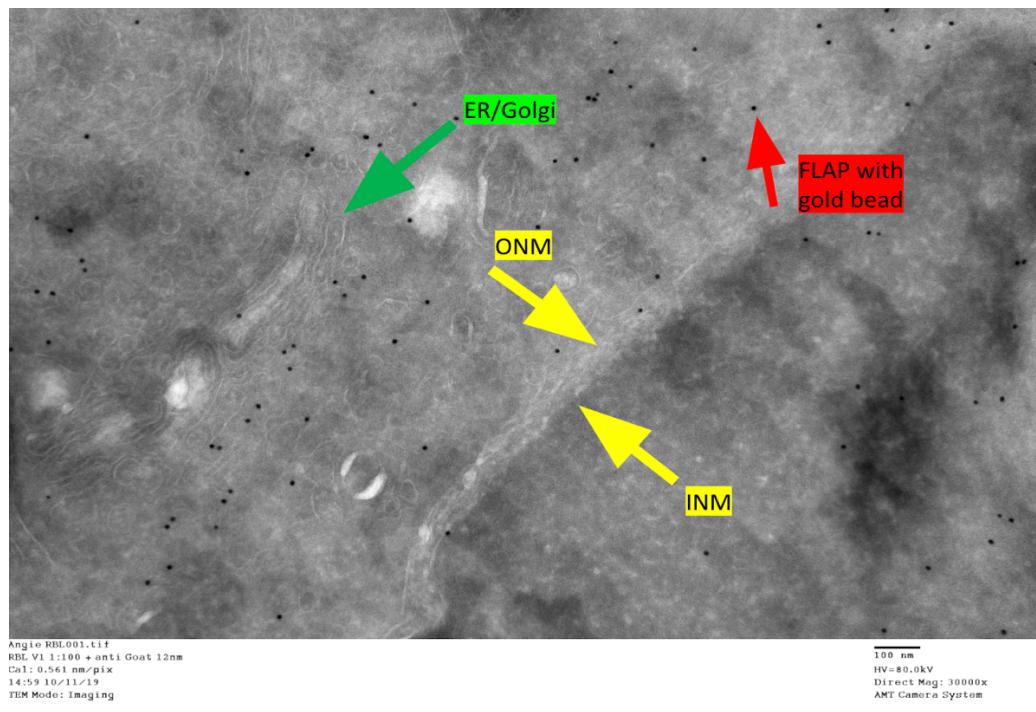
FLAPv1 RAW



FLAPv2 RBL-2H3

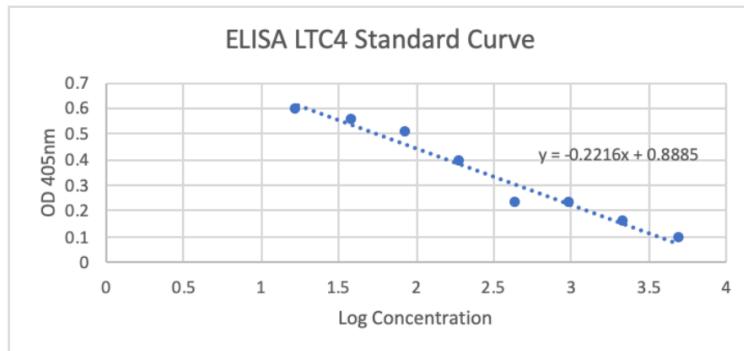


FLAPv1 RBL-2H3



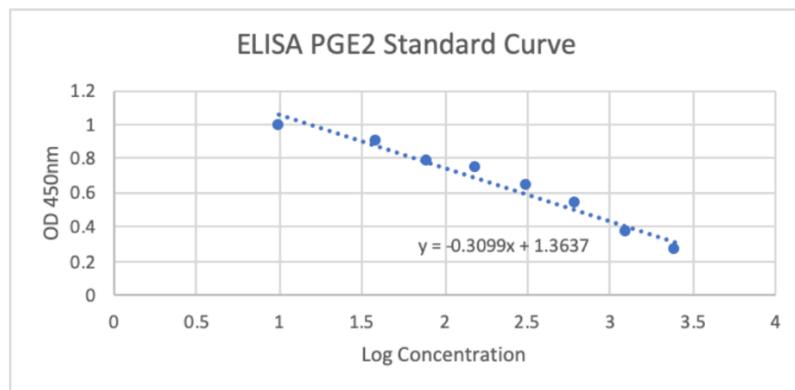
3. ELISA Results

ELISA LTC4 Results



	Average	\pm SD	Log Concentration	Concentration (pg/mL)
Control	0.7590	0.0368	0.584386282	3.840486843
Primed	0.8000	0.0156	0.399368231	2.508235042
Activated	0.2820	0.0198	2.736913357	545.6489922

ELISA PGE2 Results



	Average	\pm SD	Log Concentration	Concentration (pg/mL)
Control	0.9053	0.1934	1.479079273	30.13556046
Sample	0.2433	0.0493	3.615252232	4123.369288

Bibliography

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