MQP-BIO-JBD-

ELEXIS Based Screen of Human LIGs with ErbB1,2,4

A Major Qualifying Project Report

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

In

Biochemistry

By

_____
Andrew Brunelle

April 15, 2018

APPROVED:

_____
Joseph Duffy, Ph.D.
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

Several types of cancer have been linked to the Epidermal Growth Factor Receptor (EGFR) family of tyrosine kinases. An EGFR homolog in *Drosophila*, dEGFR, was found to be inhibited by a protein called Kekkon1 (Kek1). Kek1 is a member of the LIG super family, meaning it contains both Leucine-rich repeats (LRRs) and Immunoglobulin (Ig) domains. Based on structural similarities it was hypothesized that members of the human LIG family could interact with the human members of the EGFR family. An ELEXIS interaction assay was used to test interactions between human LIGs and the EGFRs (ErbB1,2,4). Interactions between different human LIGs were also tested. Binding between the human LIGs and receptors could indicate the potential use of LIGs as a cancer therapeutic.

ACKNOWLEDGEMENTS

I would like to thank Professor Duffy for his assistance and guidance throughout this entire process. This project would not have been possible without his previous research and knowledge of laboratory techniques. Duff taught me a variety of lab techniques and helped me to think about the project in a critical fashion.

I would also like to thank Alexander Putnam for all of his help with the experiments throughout the project. Alex taught me valuable techniques that will continue to be helpful long after this project is finished. He also provided a number of protein samples for interactions and cells for transfection.

Finally, I would like to thank all of the labs in the BBT and CBC departments who allowed me to use their equipment and reagents.

With the help of all the people involved in the MQP process I was able to develop my abilities in lab and my capacity as a scientist.

TABLE OF CONTENTS

INTRODUCTION

The human nervous system serves as a method for transmitting information throughout the body. As neural circuits develop, axons extend through the body and innervate target cells. This process of branching and neural development requires a series of receptors and their ligands to organize and regulate axonal extension. A family of molecules, known as LIGs, has been found to promote neural growth by interacting with tyrosine receptor kinases (Mandai et al. 2009).

Structural Features of LIG Proteins

LIGs are transmembrane proteins that contain both leucine-rich repeats (LRRs) and immunoglobulin domains (Ig domain). These two sequences are commonly found in proteins, but it rare to find both in a single protein. LRRs have been found to promote interaction between LRR proteins (Aylwin and Ramnik 2011). Figure 1 shows the structure of a general LIG protein with its LRRs and Ig-like domain.

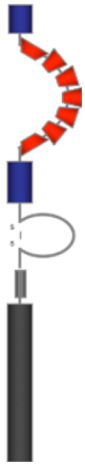

Figure 1. Structure of a LIG protein. The red trapezoids contains the LRRs and the grey loop contains the Ig-like domain.

Leucine Rich Repeats

The leucine-rich repeat is a common structural motif that is characterized by 20-30 amino acids that have a conserved pattern of eleven residues primarily comprised of leucines - LxxLxLxxNxL, where x is any residue, L is leucine,and N is asparagine (Bella et al. 2008; de Wit et al. 2011). This area forms a β-strand and a loop, and this connects back to the C-terminus (Kajava 1998). These repeats typically come in tandem with as few as three and up to as

many as 30 repeats, which are then flanked at the N and C terminus by cysteine-rich regions (Kobe and Deisenhofer 1994; Kajava 1998; Bella et al. 2008; de Wit et al. 2011). The β-strands of each repeat together form a β-sheet, creating a horseshoe-shaped structure that acts as a binding site for diverse protein-protein interactions, including between other LRR regions. The β-sheet or concave side of the horseshoe shaped typically serves as the ligand-binding site. Proteins in the LRR family have been found to be involved in diverse events, including cell signaling pathways, hormone-receptor interactions, and the connectivity of neural circuits (de Wit et al. 2011).

Immunoglobulin domains

In contrast to LRRs, Immunoglobulin (Ig) domains were defined initially as specific conserved sequences found in antibodies or immunoglobulins. Ig domains are found in a large number of proteins, broadly defined as the Ig Superfamily. These domains are composed of ~100 amino acids in which two sheets of antiparallel β-strands linked by loops create a sandwich-like structure (Williams and Barclay 1988). The loops are connected through disulfide bridges (Williams and Barclay 1988). Similar to interactions between LRRs, Ig domains have also been found to be involved in binding with other axonal proteins with Ig domains (Brümmendorf and Rathjen 1996). Taken together these observations support the notion that in addition to interacting with other molecules, LIG proteins may in fact interact with each other.

The LIG superfamily can be divided into subfamilies based on their structural organization and sequence relationships (Homma et al. 2009). This project focused on two different

subfamilies of LIGs known as the LRITs and NLRRs (Ishii et al., 1996; Sheikh et al., 2016; Sarria et al. 2018; Ueno et al. 2018). There are three proteins in the LRIT family: LRIT1, LRIT2, and LRIT3 (Ueno et al. 2018). LRIT1 contains 7 LRRs and 1 Ig-like domain, and has been identified as a retinal transmembrane protein that regulates light adaptation and daylight vision (Sarria et al. 2018; Ueno et al. 2018). LRIT2 contains 6 LRRs and 1 Ig-like domain, and its exact function is still being studied. LRIT3 contains 6 LRRs and 1 Ig-like domain, and was found to interact with the fibroblast growth-factor receptor (FGFR) (Kim et al. 2012). Overexpression of the FGFRs has been linked to cancer, and LRIT3 was found to regulate the FGFR1 signaling pathway by facilitating the exit of FGFR1 from the ER (Kim et al. 2012). Like the LRITs, there are three members of the NLRR family (NLRR1,2,3), however unlike the LRITs, which include a fibronectin domain in their extracellular region, the NLRRs only include the LRRs and an Ig domain (Ishii et al., 1996; Sheikh et al., 2016; Sarria et al. 2018; Ueno et al. 2018). Currently, the NLRRs have been implicated in neuroblastoma, but significantly less is known about this LIG subfamily.

Despite being structurally similar to each other, current evidence across the LIG family suggests family members can serve a variety of distinct molecular and cellular functions, including roles in receptor tyrosine kinase (RTK) signaling and synaptic signaling. The focus of this project will be on specific LIGs and their ability to interact with receptor tyrosine kinases, or RTKs. Specifically, this project will look at interactions between members of the human LIG family and a family of RTKs known as the Epidermal Growth Factor Receptors (EGFRs).

Epidermal Growth Factor Receptors

Epidermal Growth Factor Receptors, or EGFR/ErbB, are a family of human receptor tyrosine kinases that induce cell differentiation and proliferation (Voldborg et al. 1997). There are four ErbB receptors that have been found in humans – EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4 (Fig. 2). The ErbB receptors are known for their role in cancer proliferation, including neuroblastoma and breast cancer (Zhang et al. 2007). The ErbB receptors are composed of an intracellular domain, a short transmembrane domain, and an extracellular domain with ligand-binding activity (Normanno et al. 2006). The extracellular region contains four subdomains numbered I-IV. Ligand binding takes place in the sequence related subdomains I and III. In contrast, domains II and IV are involved in inter-receptor interactions. The intracellular domain is highly conserved across the ErbB receptors and is composed of a tyrosine kinase domain and the C-terminal tail (Normanno et al. 2006). The structure of the ErbB receptors allows the ligand binding to induce an intracellular response.

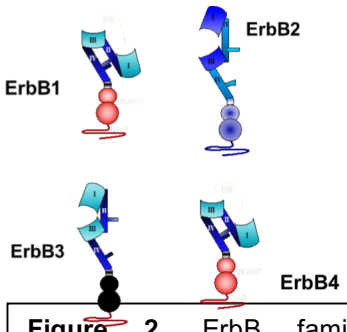

Figure 2. ErbB family. Domains I and III of ErbB2 are blue to indicate they do not bind ligands. The intracellular domain of ErbB3 is black to indicate that there is no kinase activity.

ErbB receptors can be activated by a variety of ligands and ErbB-specific growth factors. There are three groups of ligands that bind the ErbB receptors: epidermal growth factor (EGF), amphiregulin (AR), and transforming growth factor alpha. Not all of the ErbB receptors have the same domains; ErbB2 does not have the ability to bind ligands, so its main purpose is to dimerize with the other ErbB receptors. ErbB3 does not have an

intracellular tyrosine kinase domain (Normanno et al. 2006). The structure of each ErbB receptor can be seen in Figure 2.

Upon binding a ligand, ErbB receptors will dimerize in order to activate their intracellular kinase domain. The tyrosine residues on the receptor are phosphorylated to facilitate protein binding, and the protein binding induces a cellular response (Fig. 3).

Ligand binding induces a conformational change in the ErbB receptors that allows the II and IV regions of the receptors to dimerize using a dimerization loop (Lemmon et al. 2014). Until the receptor binds a ligand the dimerization loop is hidden and the receptors are predominantly monomeric.

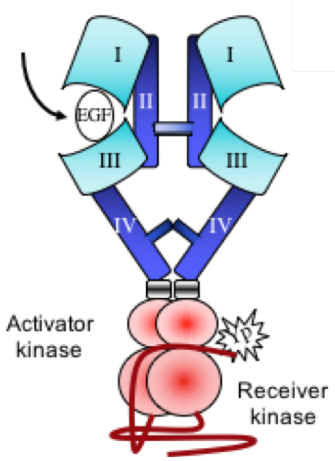

Figure 3. ErbB dimerization

Previous work from the Duffy lab had demonstrated that the *Drosophila* EGFR (dEGFR) is negatively regulated by the LIG Kekkon1 (Kek1) through direct binding (Alvardo et al. 2004). With this interaction in mind, the goal of this project was to assess if specific human LIGs interact with members of the human EGFR family.

MATERIALS AND METHODS

Generating LIG Constructs for Bait and Prey Proteins

For the ELEXIS interaction assay secreted variants of the human LIGs that were tagged had to be generated. Gateway cloning was used for all cloning steps. Two types of C-terminal fusions were generated, fusions to Alkaline Phosphatase represented the Bait constructs, while fusions Fc represented the Prey constructs. LIG constructs, including LRIT1, LRIT2, LRIT3, TrkB, and TrkC, were initially designed *in silico* using Gene Construction Kit (GCK) software. First, the extracellular region for each LIG was predicted using the online transmembrane prediction software CCTOP (http://cctop.enzim.ttk.mta.hu). Based on this a 5' attB1.1 primer containing the start codon and a 3' attB2.1 primer corresponding to the region prior to the transmembrane domain were designed for LRIT1, LRIT2, LRIT3, TrkB, and TrkC. Constructs and primers for NLRR1,2,3 had been previously designed in the lab. The appropriate primer pairs were then used in PCR reactions using the corresponding human LIG cDNA as a template, to amplify the portion of the open reading frame representing the sLIG variant. The PCR fragments were digested and amplified by PCR. PCR fragments were then analyzed by gel electrophoresis and purified using Qiagen gel extraction kits. PCR fragments were then subcloned by a BP Gateway™ (Invitrogen) reaction to form an entry clone, or pENTR. These pENTR constructs were sequenced (Eton BioScience) and confirmed using the Sequencher analysis program. Correct pENTR clones were then subjected to a LR Gateway™ (Invitrogen) reaction to shuttle the human LIG extracellular coding sequence into the pIB-attB-AP or pIB-attB-Fc vector to produce an expression

clone with the appropriate sLIG variant and the correct tag. Standard molecular techniques were used to transform and recover plasmids. Expression clones were confirmed by 5' and 3' sequenced (Eton BioScience) and confirmed using the Sequencher analysis program. Cloning procedure is diagrammed below in Figure 4.

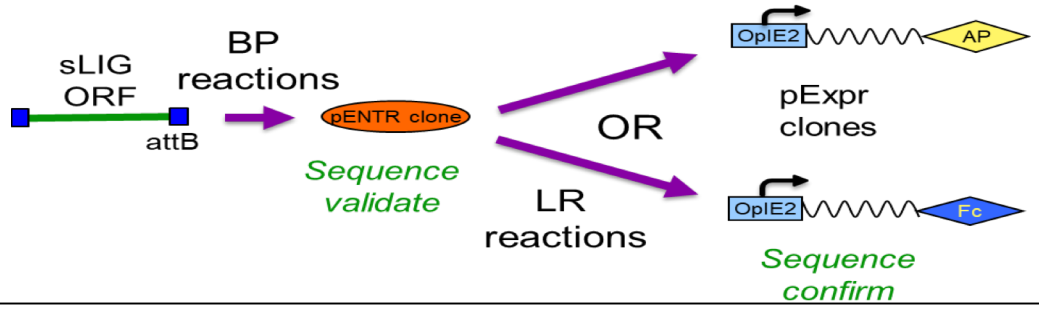

Figure 4. pIB system used in transfection with AP or Fc-tagged LIG constructs for protein expression.

Transfection of LIG Expression Clones

To produce all sLIGs, expression constructs were transiently expressed in *Drosophila* S3 cells. S3 *Drosophila* cells were thawed and maintained as described by *Cherbas, et al* (Cherbas, 1998). To transfect cells with the DNA constructs, cells were first counted to 3.125 x 10^6 cells/ml and 1.6 ml of cells were seeded in 6-well cell culture plates. The seeded cells were incubated overnight under normal growth conditions of 25°C without CO_2 to obtain 100% confluence. The cells were then transfected with the respective LIG pIB responder constructs (NLRR2-AP, NLRR3-AP, TrkB-Fc, TrkC-Fc, LINGO1-AP, and LINX-AP) following the protocol described by *Wojtowicz et al.* (Wojtowicz, et al. 2007).

After incubating the transiently transfected cells for 1 week, the media supernatant was collected and gently centrifuged to pellet any suspended cells. The supernatant was

collected, filtered with a 0.22 μm PES filter, vortexed, and aliquoted into eppendorf tubes. Each protein sample was then stored at 4° C.

Bait Assessment

Each AP bait sample was assessed by hPLAP enzymatic activity against an hPLAP enzymatic standard curve, previously generated by the Duffy lab. The standard curve was created by making a dilution series of 100 U/L, 75 U/L, 50 U/L, 25 U/L, 10 U/L hPLAP in cell culture supernatant. The activity of each AP bait sample was assessed against the standard curve by comparing a high concentration, 75 μL, and a low concentration, 20 μL. The activity of each sample at each concentration against the activity of cell culture supernatant was assayed by adding an equal volume of PNPP (Pierce) substrate to both the hPLAP standards and AP-tagged protein samples and tracked every minute over a 20 minute time frame at 405 nm using a Victor3 plate reader and Wallac software. The velocities of each AP bait sample concentration over 10 minutes were converted into a standard curve assessing hPLAP velocity vs. hPLAP concentration (U/L) that was used to quantify the hPLAP concentration in each AP bait sample. These velocities were used to quantify and normalize the AP-tagged protein samples against the hPLAP standard curve.

Prey Assessment

Each Fc prey sample was assessed using an Fc ELISA (Syd Labs) against an Fc standard (Jackson Immuno). An Fc standard was tested at 5 ng/mL, 0.313 ng/mL, and 0 ng/mL. The Fc samples (TrkB-Fc and TrkC-Fc) were each tested at two dilutions, 1:250

and 1:750. An equal amount of TMB Ultra (Thermo) was added to each well and was tracked over a 13 minute time frame at 590 nm using a Victor3 plate reader and Wallac software. The velocities of each sample were compared to the HRP standard curve in order to quantify the protein concentration in each sample.

Protein Interactions

To determine if AP-tagged LIGs interact with Fc-tagged ErbB family of receptors or the TrkB,C-Fc receptors, an ELISA based screening assay described by *Wojtowicz et al*. was used (Wojtowicz, et al. 2007). This enzyme linked extracellular interaction assay, or ELEXIS, was set up following Figure 5 and is described in detail below.

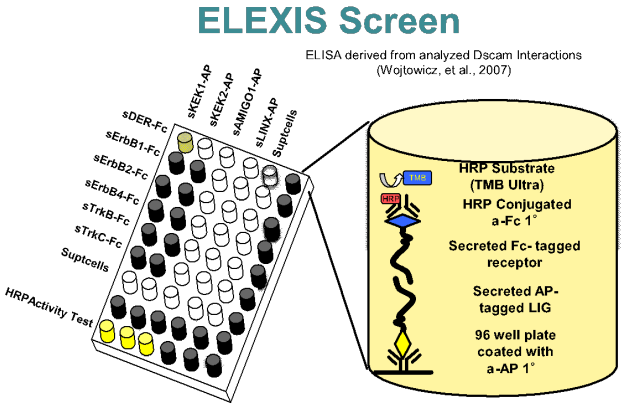

Figure 5. Structure of an ELEXIS assay.

Each interaction well on a Medisorp plate was incubated with a mixture of 3 µg/mL Ms-anti-AP (8B6.18 Thermo) in 1x PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl) overnight at 4° C on a rocking platform. Wells were washed 4x1min with 300 µl of wash buffer PBST (1x PBS and 0.05% Tween20) at room temperature on a rocking platform. 400 µl of Casein block solution (2.25% Casein in 1x PBS) was added to each well and incubated for 1.5 hours at room temperature on a rocking platform. Each interaction reaction was generated during the blocking incubation period and contained 0.5 pmol of each Fc-tagged interacting prey sample, 0.45 pmol of the AP bait sample, a final concentration of 1 µg/mL of the HRP conjugated Ms-anti-Fc detection antibody, and

supernatant to a total volume of 50 µL. The block solution was removed and the interaction mix was added into each well, the plate covered with tinfoil and incubated for 4 hours at room temperature on a rocking platform. After the incubation period wells were washed 4x1 min with 300 µl of PBST at room temperature on a rocking platform protected from light. The presence or absence of an interaction was assessed by adding 100 µL of 1-Step TMB Ultra HRP Substrate (Pierce) to each well. Wells were then tracked at 590nm every 5 minutes for 25 minutes at room temperature using a Victor3 plate reader and Wallac software. The reactions in each well were stopped with 100 µL of 1 M Phosphoric Acid (H_3PO_4) stop solution and the endpoint absorbance was detected at 450 nm. Each protein interaction was compared to the positive control interaction between sKek1-AP and sdEGFR-Fc, with the negative control sKek2-AP and sdEGFR-Fc, which fails to exhibit an interaction. Background binding for both the Bait and Prey were assessed by comparing the interaction to both AP-tagged bait with supernatant and Fc-tagged prey with supernatant. Relative interaction signals were generated by the following formula: ((Bait•Prey abs.) – Prey•Supt. abs.))/ (Bait•Supt. abs.).

RESULTS

As noted in the introduction, Kek1 binds directly to the *Drosophila* EGFR. Recent work in the Duffy lab has recently demonstrated this interaction can be recapitulated using secreted versions of the molecules in the ELEXIS assay developed by *Wojtowicz et al*. (Wojtowicz, et al. 2007). Using this assay, interactions between human LIGs and the EGFR family, as well as between human LIGs were analyzed.

Generating Bait and Prey sLIG variant constructs:

To test for interactions using the ELEXIS assay, prey and bait constructs encoding secreted versions of the human LIGs and EGFR family members were generated. Fc-tagged prey constructs for ErbB1, 2, 3, and 4 had previously been generated by A. Putnam. In addition, the LIGs, TrkB and TrkC, which are also RTKs due to the presence on their intracellular kinase domains, were also tagged with Fc and used as preys. In contrast, expression constructs encoding the human LIGs, LRIT1, LRIT2, LRIT3, NLRR2, and NLRR3 were initiated. For NLRR2 and 3 both AP and Fc expression constructs were generated, while the those for LRIT1,2 and 3 are currently in progress.

Protein Expression and Quantification

Once the expression constructs were generated, protein expression for the tagged Baits and Preys was performed as described in the materials and methods. Briefly, expression clones were transiently transfected into S3 *Drosophila* cells, supernatants recovered and assessed either for AP activity (Baits) or the presence of the Fc tag (Preys).

Bait – AP tagged LIG protein concentration was assessed using an AP enzymatic assay. The assay used PNPP substrate and AP-tagged LIGs to test the absorbance of a known volume of the LIGs. The absorbance of the samples was compared to an hPLAP standard curve to determine the concentration of the AP-tagged LIG. Figure 6 shows a photograph of the AP-enzymatic test plate. Figure 7 is a graph of the hPLAP standard absorbance. In Figure 8 the absorbance of each AP-tagged LIG was graphed comparing absorbance over time. Using this data, it was determined that the concentration of sLINX-AP was 893.0 µU/µL and the concentration of sLINGO1 was 64.0 µU/ µL. Based on the absorbances for sNLRR2 and sNLRR3 they did not appear to express enough protein to be quantified.

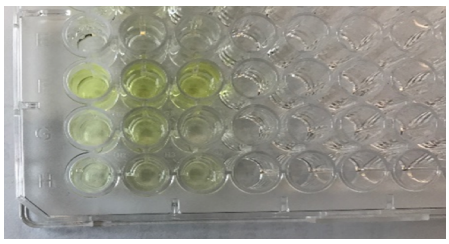

Figure 6. Bait quantification. Photo image of the 96 well plate used for AP-tagged protein quantification. Rows 1 and 3 contain experimental samples, while rows 2 and 4 contain AP standards, all at low and high concentration, respectively.

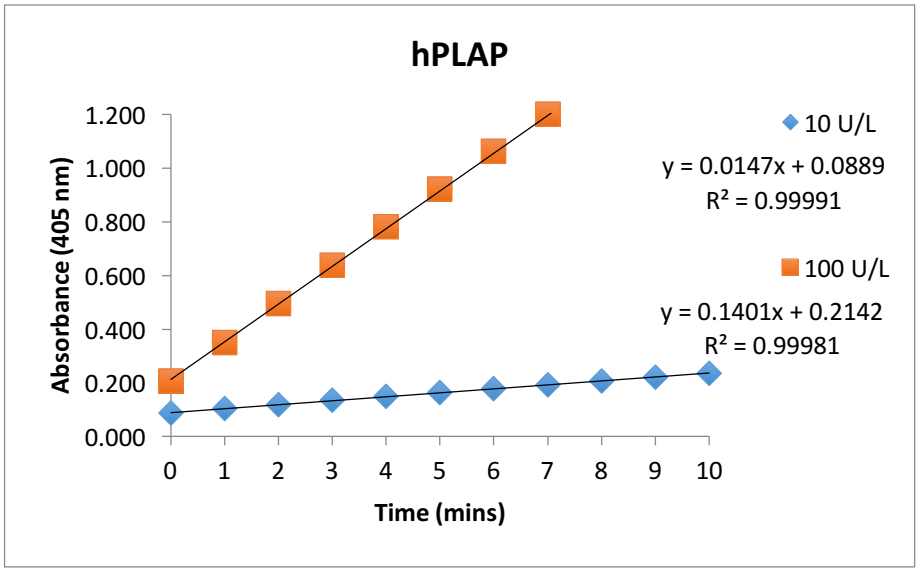

Figure 7. hPLAP standard curves at 10 U/L and 100 U/L over 10 minutes.

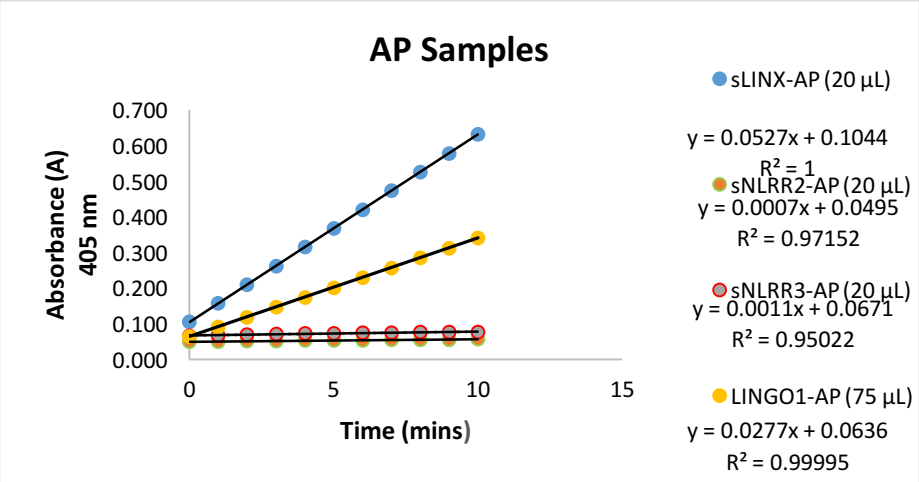

Figure 8. Activity plot over 10 minutes for sLINX-AP, sNLRR2-AP, sNLRR3-AP, and LINGO1-AP. sLINGO1-AP is at a higher concentration than the other samples.

Prey – Fc tagged LIGs (sTrkB-Fc and sTrkC-Fc) were quantified using an Fc-ELISA. The assay used a coating and detection antibody solution (Syd Labs) to anchor and detect the Fc-tagged proteins and an Fc-standard (Jackson Immuno) to compare the sample absorbance to a known value as detailed in the Materials and Methods. dEGFR-Fc was also run as a control (quantified previously in the Duffy lab). After adding the TMB Ultra, absorbance of the samples was measured at 590 nm every minute for 13 minutes. Figure 9 is a photo of the ELISA plate after 13 minutes. The data for the standards can be seen in Figure 10 and the experimental data for sTrkB-Fc and sTrkC-Fc can be seen in Figure 11. The reaction was stopped after 13 minutes by the

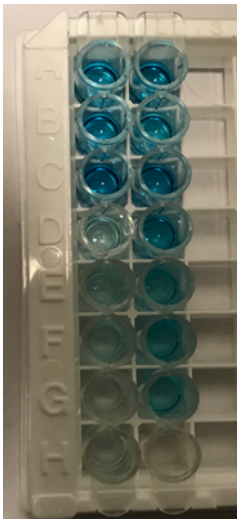

Figure 9. Photo of Fc-ELISA plate after 13 minutes. The left column contains the high and low standard in triplicate. The right column contains a 1:250 and 1:750 dilution of TrkB-Fc, TrkC-Fc, and dEGFR-Fc.

addition of 1M H_3PO_4 (Sigma). Based on the ELISA assessment of the Prey proteins, sTrkB-Fc had a final concentration of 4.0 ng/µL, sTrkC-Fc had a final concentration of 4.6 ng/µL, and sdEGFR-Fc had a final concentration of 3.8 ng/µL.

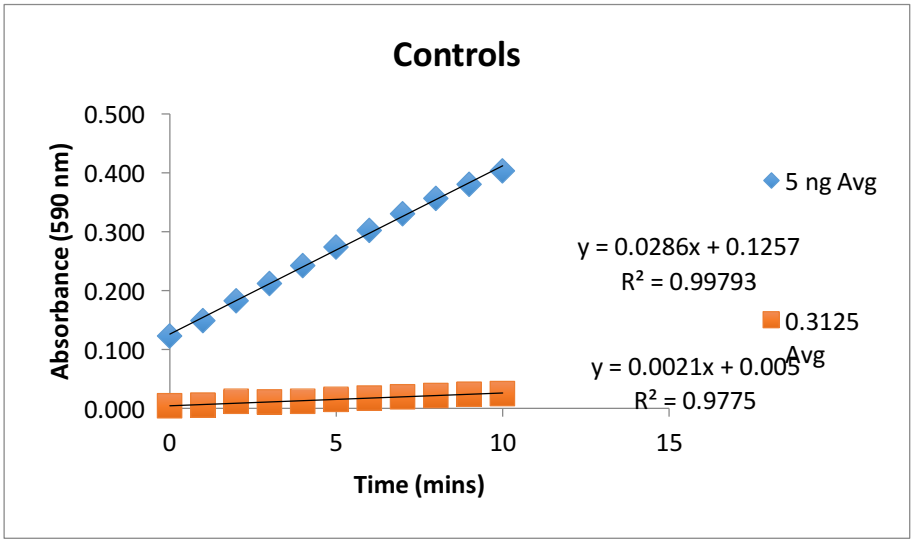

Figure 10. Absorbance over time for a high and low concentration of the HRP Fc-Standard.

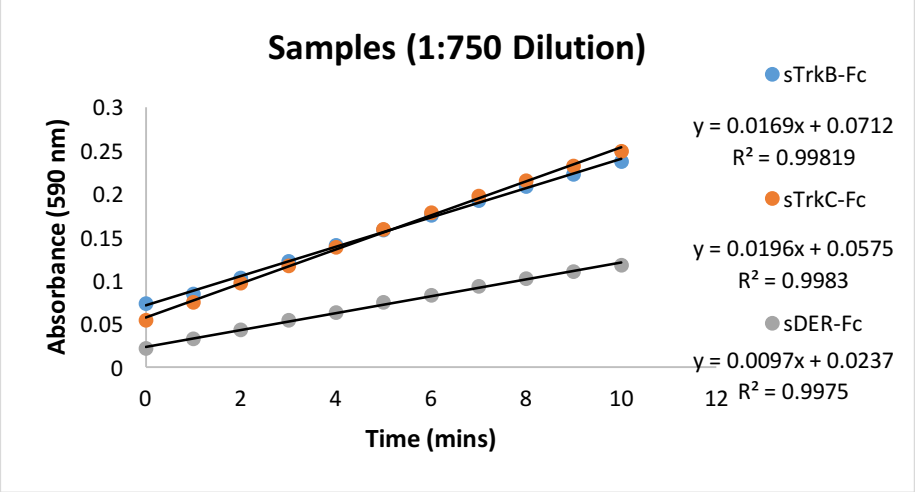

Figure 11. Absorbance over time for Fc-tagged LIGs.

Testing LIG - EGFR Family and LIG - LIG Interactions

While the sLRIT1,2,3 clones were not complete and expression of the sNLRR1,2 AP tagged clones was not recovered, expression of sTRKB-Fc, sTRKC-Fc and sLINX-AP were obtained as described above. These along with sAMIGO1-AP were tested in an ELEXIS assay to determine their binding properties. Specifically, sLINX-AP and sAMIGO1 were tested with the receptors sErbB1-Fc, sErbB2-Fc, sErbB4-Fc, sdEGFR-Fc, and the LIGs/receptors TrkB-Fc, and sTrkC-Fc. The sKek1/sdEGFR interaction served as a positive control and sKek2/sdEGFR interactions served as a negative control. sKek1-AP, sKek2-AP, sAMIGO1-AP, sdEGFR-Fc, sErbB1-Fc, sErbB2-Fc, and sErbB4-Fc were provided by Alex Putnam from the Duffy lab. Figure 12 is a photo of the ELEXIS plate after stopping the detection reaction. Figure 13 shows the extracellular protein-protein interaction (ePPI) signal representing the fold interaction of each sample compared to their readings above the background (see materials and methods for description). In the ELEXIS assay, the only combination that exhibited an interaction was the positive control of sKek1/sdEGFR. In contrast, none of the other sLIG-sReceptor or sLIG-sLIG combinations resulted in a positive signal.

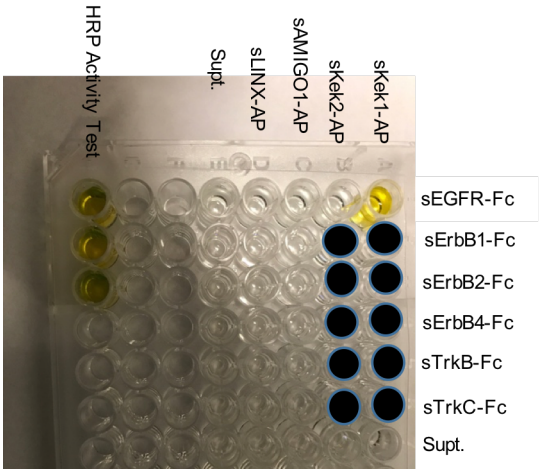

Figure 12. Photo of the ELEXIS assay. Yellow color indicates an interaction. Black wells were not used. Positive control in A1 and negative control in B1.

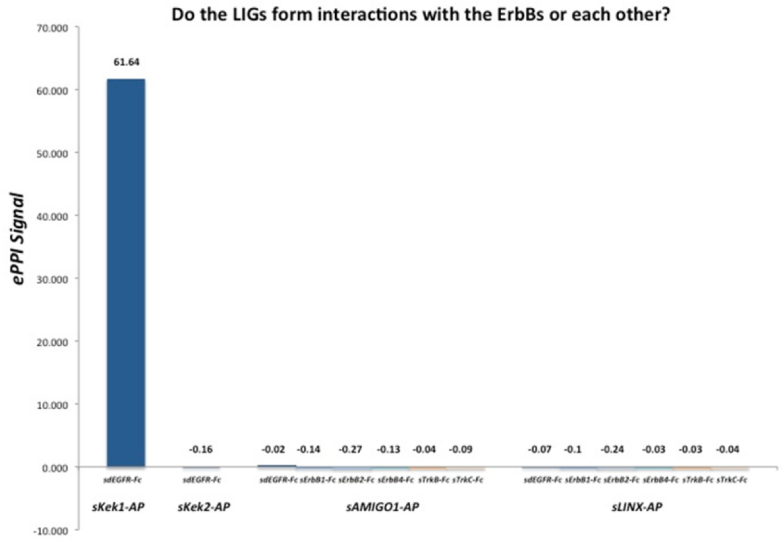

Figure 13. sAMIGO1 and sLINX ePPI signals compared to the positive (sKek1/sdEGFR) and negative (sKek2/sdEGFR) controls.

DISCUSSION

Kek1 is known to bind to dEGFR in a variety of contexts including the ELEXIS assay. In addition, previously published work reported that full-length LINX and AMIGO1 were able to bind to full length TrkC (Mandai et al. 2009). This led to the objective of this report to determine if an interaction between human LIGs and members of the EGFR family could be detected in the ELEXIS assay.

In this report, data indicating that of four of the six LIGs that were transfected into *Drosophila* cells were successfully secreted, but only three, sLINX-AP, sTrkB-Fc, and sTrkC-Fc were in concentrations high enough to continue with the interaction assay. sLINGO1 would need to be concentrated or transfected again in order to produce enough product to test interactions. sNLRR2 and sNLRR3 did not produce quantifiable product

after being transfected, and would need to be transfected again to obtain a usable concentration.

After the proteins were assessed, sLINX-AP and sAMIGO1-AP were run against sErbB1,2,4-Fc, sTrkB-Fc, and sTrkC-Fc. A positive and negative control were run on the same plate to ensure that the assay functioned as intended. The positive control gave high signal, ePPI of 61, while the negative control gave little to no signal. This confirmed that the assay format is functional. Little to no signal was detected for all of the other interactions, suggesting that, in this format, sLINX-AP and sAMIGO1-AP do not bind to sErbB1, sErbB2, sErbB3, sTrkB-Fc, or sTrkC-Fc. While this initial screen did not uncover any interactions, it will be important to assess a number of key factors. The absence of any detectable interactions between the LIGs and receptors, could be for a number of reasons.

First, it is possible that the lack of interaction between the AP-tagged sLIGs and Fc-tagged sReceptors was due to the proteins not folding properly because the transmembrane and intracellular domains were removed, or second that any such interactions require these regions as well. Thirdly, while expression of the AP and Fc tags was detected, it is possible that proteolysis during expression resulted in separation of the tag from the Bait or Prey molecules. To determine if proteolysis occurred, next steps would be to perform a Western blot to characterize if the tagged proteins are the expected sizes. Finally, if positive controls could be established for either sLINX or sAMIGO1 or the sEGFR or Trk family members this would provide greater confidence that the absence of an interaction was not simply due to inappropriate folding.

REFERENCES

Bella, J., et al. (2008). Leucine Rich Repeat. Cellular and Molecular Life Sciences 65 (5), 2307–2333.

Brümmendorf, T., & Rathjen, F. G. (1994). Cell adhesion molecules. 1: immunoglobulin superfamily. *Protein Profile*, 1(9), 951–1058.

de Wit, J., Hong, W., Luo, L., & Ghosh, A. (2011). Role of leucine-rich repeat proteins in the development and function of neural circuits. *Annual Review of Cell and Developmental Biology*, 27, 697–729.

Homma, S., Shimada, T., Hikake, T. and Yaginuma, H. (2009). Expression pattern of LRR and Ig domain-containing protein (LRRIG protein) in the early mouse embryo. *Gene Expr. Patterns* 9 (1), 1-26.

Kajava, A. V. (1998). Structural diversity of leucine-rich repeat proteins. *Journal of Molecular Biology*, 277(3), 519–527.

Kobe, B. and Deisenhofer, J. (1994). The leucine-rich repeat: a versatile binding motif. *Trends Biochem Sci* 19, 415-21.

Mandai, K., Guo, T., St Hillaire, C., Meabon, J. S., Kanning, K. C., Bothwell, M., & Ginty, D. D. (2009). LIG family receptor tyrosine kinase-associated proteins modulate growth factor signals during neural development. *Neuron*, 63(5), 614–627

Nobuko, I., Wanaka, A., and Tohyama, M. (1996). Increased expression of NLRR-3 mRNA after cortical brain injury in mouse. *Molecular Brain Research 40 (1)*, 148-152.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M. R., Salomon, D. S. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene*, 366(1), 2–16.

Ng, A., and Ramnik J. X. (2011). Leucine-Rich Repeat (LRR) Proteins: Integrators of Pattern Recognition and Signaling in Immunity. *Autophagy* 7 (9), 1082–1084.

Sarria, I., Cao, Y., Wang, Y., et al., (2018). LRIT Modulates Adaptive Changes in Synaptic Communication of Cone Photoreceptors. *Cell Reports* 22, 3562-3573.

Sheikh, A., Takatori, A., Hossain, S., et al., (2016). Unfavorable neuroblastoma prognostic factor NLRR2 inhibits cell differentiation by transcriptional induction through JNK pathway. *Cancer Science* 107, 1223-1232.

Ueno,A., Omori, Y., Sugita, Y., Watanabe, S., et al., (2018). Lrit1, a Retinal Transmembrane Protein, Regulates Selective Synapse Formation in Cone Photoreceptor Cells and Visual Acuity. *Cell Reports* 22, 3548–3561.

Voldborg, B.R., Damstrup, L., Spang-Thomsen, M. and Poulsen H.S. (1997). Epidermal Growth Factor Receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Ann. Oncol*. 8(12). 1197-1206.

Williams, A. F., & Barclay, A. N. (1988). The immunoglobulin superfamily--domains for cell surface recognition. *Annual Review of Immunology* 6, 381–405.