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Interaction Assay for LIG Superfamily Proteins

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Table of Contents

Abstract

The LIG superfamily is characterized by its unique extracellular domain structure which contains a series of leucine-rich repeats (LRRs) followed by one or more Immunoglobulin (Ig) domains. Knowledge about biological pathways regulated by LIG family members is still at its infancy, though some members have been associated with novel biological functions such as inhibition of epidermal growth factor receptor (EGFR) in *Drosophila* and neuronal regeneration in humans. The goal of this project was: to design a high-throughput protein interaction assay to rapidly identify potential binding partners for LIG family members that may provide insight to their biological function.

Acknowledgement

I would like to thank Duff first and foremost for guiding me through my four years at WPI. By training me into a better scientist, you have also helped me become a better person. In the past, I have always operated with the fear to fail but you have taught me that whether it is running an experiment or facing a new challenge life, I should be willing to go out there and get my hands dirty. Failure is inevitable in many cases but what is more important is to learn from my mistakes. Adopting this new mentality has helped me gain more relief and confidence in my research and also in my personal life.

I would also like to thank Prachi Gupta for being my first mentor in Duffy Lab. I flipped my first flask of *Drosophila*, set up my first PCR, poured my first gel, and ran my first electrophoresis with you. Thank you for being warm and patient with me and for teaching me important lessons of Research 101.

To Harita Haridas, I'm sure you are the one who has witnessed the most of my silly mistakes. Thank you for always being there and at the most absurd hour to help me with troubleshooting. I'm so grateful of you teaching me how to perform midiprep, how to streak my first plate, how to organize my poster and countless other things.

I also want to thank Michelle Arata for instructing me the basic steps of cell culture and Western Blot. You made me start believing that with hard work and practice, I could improve my skills as a scientist.

Thank Professor Camesano for allowing me to use your microscope. Thank Melissa Mobley and Karen Tran for your kind words of encouragement. Thank Abbie White for letting me use your TMB. Thank Denis Kole for teaching me how to perform ELISA.

Finally, I would like to extend an enormous thank to Professor Gericke for advising me on the biochemical aspects of my project.

With all of your help and support, I reckon I have walked a long way from an immature freshman to a senior waiting for what is ahead of her with enthusiasm and optimism.

Background

Cell-to-cell communication is critical for the survival and development of multicellular organisms. Development from a fertilized egg to a complete organism requires communication and interaction among billions of cells. Research in the area of cell signaling have helped provide some answers to the most important questions in biology, such as how cell fates are determined and how different types of cancer progress. Work in the Duffy lab has been involved in investigating cell-to-cell communication. One of the most important findings discovered by the lab is the function of the *Drosophila* transmembrane molecule Kekkon 1 (Kek1) in a negative feedback loop to regulate the activity of the Epidermal Growth Factor Receptor (EGFR) during oogenesis (Ghiglione et al., 1999).

Overview of Epidermal Growth Factor Receptor (EGFR) Family

EGFR or ErbB is the first cell-surface receptor to be linked directly to cancer. Following the identification of EGFR in humans, three additional members of the receptor family were discovered including ErbB2, ErbB3, and ErbB4. The general structure of EGFR and other family members consists of a heavily glycosylated extracellular region with 4 domains (I-IV), a transmembrane domain, and cytoplasmic tyrosine kinase domain (*Figure 1*) (Burgess et al., 2003).

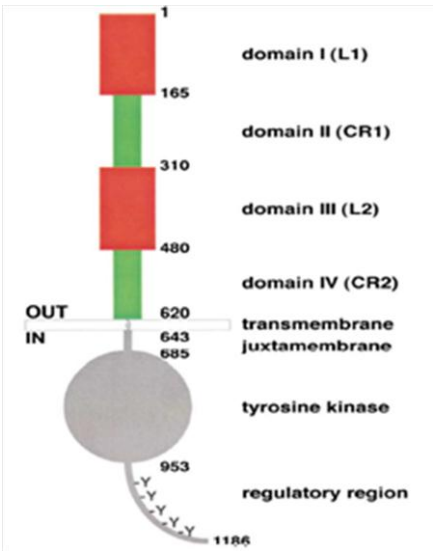

Figure 1: Domain Organization of Epidermal Growth Factor Receptor (Burgess et al., 2003)

Ligand binding to the extracellular domain of EGFR via contacts in domain I and III locks the receptor in an active conformation and exposes the dimerization arm in domain II. Subsequent receptor homo- or hetero- dimerization triggers transphosphorylation of a specific subset of tyrosine residues in the C-terminal tail (*Figure 2*). A variety of cytoplasmic kinase cascades, such as mitogen-activated protein kinase (MAPK), can follow (Schlessinger, 2002).

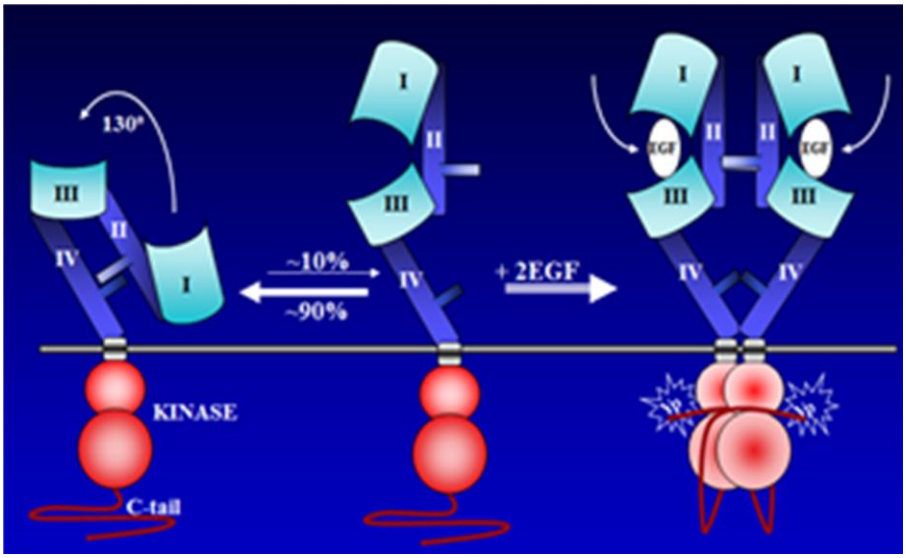

Figure 2: Mechanism of Activation of Epidermal Growth Factor Receptor

Distinct among four human ErbB receptors, ErbB2 has no known soluble ligand to control its activity. Its structure reveals domain II in an "inactive" conformation and direct interactions between domain I and III in the extracellular region, which keep the receptor in open conformation and prevent ligands from binding (Burgess et al., 2003). Due to its open configuration, ErbB2 is regarded as an 'auto-activated receptor' that can transform cells and cause cancer simply through overexpression. Sequence analyses show similarity in sequence and overall domain arrangement between the single EGFR family member present in *D. melanogaster* - dEGFR/DER and human EGFR family members. For example, ErbB2 and dEGFR share 35% sequence identity over domains I-IV (Alvarado et al., 2009). The one major exception to the structural and sequence similarity is in the presence of an extra domain, termed domain V, in the extracellular region of the

Drosophila receptor. Morever, recent structural studies on dEGFR revealed that even though its activity is ligand dependent like the EGFR, its structure is actually more closely related to that of ErbB2. This surprising result indicating that the *Drosophila* receptor is in an open configuration suggests that the *Drosophila* receptor may require additional levels of regulation to ensure activity remains ligand dependent.

Kek1/dEGFR Interaction

As mentioned above, dEGFR is the ErbB2 homolog in *Drosophila*. The receptor's activity, regulated by both stimulatory and inhibitory molecules, plays a key role in many developmental decisions such as dorsal-ventral polarity, segmental identity, cuticle production and cell growth. Kekkon 1or Kek1, a *Drosophila* transmembrane molecule, can form a complex with dEGFR and inhibits this tyrosine kinase pathway in multiple tissues. Kek1 is the founding member of the Kekkon family, a set of six transmembrane proteins in *Drosophila* and a sub-division of LIG superfamily. LIG proteins' extracellular regions are characterized by leucine-rich repeats (LRR), an amino (N) and carboxyl (C) cysteine rich region flanking the LRRs, and a C2-like immunoglobulin (Ig) domain (*Figure 3*).

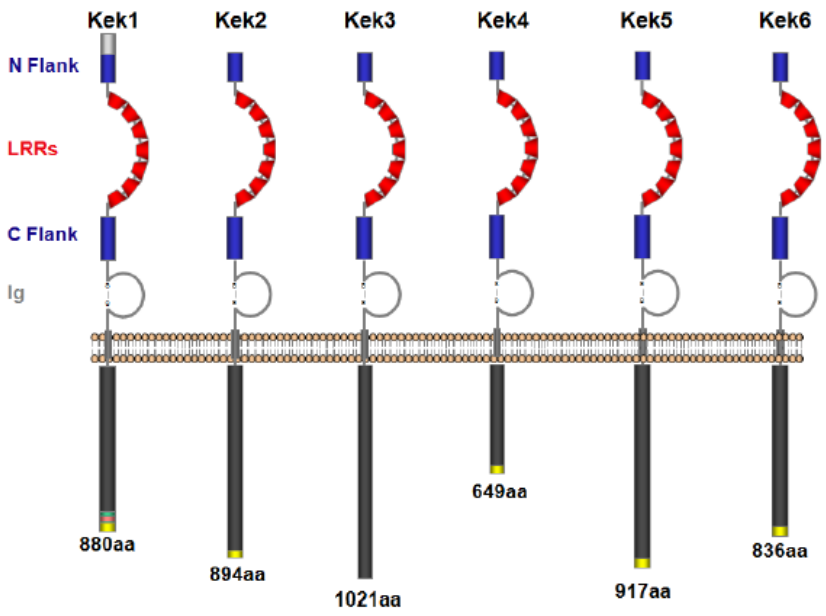

Figure 3: Schematic structures of LIG family members in *Drosophila*

Kek1 null mutations are associated with an increase in dEGFR signaling and can compensate for a decrease in receptor activity during oogenesis (Ghiglione et al., 1999). The Kek LRR domain is sufficient for dEGFR binding *in vitro*, but the juxta/transmembrane (jt/tm) portion of the molecule is critical for inhibition *in vivo*, as demonstrated by non-functional secreted forms of Kek1 and domain swaps (Alvarado et al., 2004). Domain swaps containing the Kek1 LRRs in a Kek2 backbone are inactive even though membrane tethered through the Kek2 transmembrane domain. This suggests the LRR-mediated inhibition of dEGFR is a bipartite process: the LRRs direct binding to the receptor, while the jt/tm domain ensures inhibition (*Figure 4)*.

In addition, mutational analyses with the SOK alleles in dEGFR, mutations centered in Domain V, impair the receptor's association with Kek1, suggesting an important role for this Domain in mediating regulation by Kek1 (Alvarado et al., 2004). It

is interesting to note that Kek1 has been reported to be associated with not only dEGFR but also all human ErbBs, although more recent data does not support its ability to bind to the human ErbBs (Ghiglione et al., 2003; J. Duffy, unpublished).

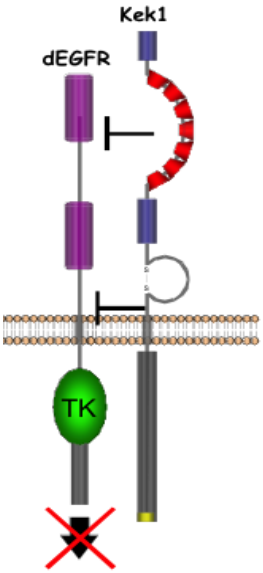

Figure 4: Kekkon 1 inhibits EGFR in a bipartite mechanism

Within the Kek family, the ability to associate with and block dEGFR's activity is unique to Kekkon 1. Similar inhibitory effects are not witnessed with Kek2, Kek3 Kek4, Kek5 and Kek6, other members of the Kek family (Alvarado et al., 2004). Of these other family members functional data is only reported for Kek5, which appears to modulate Transforming Growth Factor-β (TGF-β) signaling (Evans et al., 2009). Thus, existing data suggest that LIG family members in *Drosophila* have distinct functions but likely affect different modes of intercellular communication.

LIGS

Transmembrane molecules with LRRs and Ig domains in the extracellular region are not unique within the LIG family of *Drosophila*. There have been 36 human LIG proteins discovered: four LINGO, three NGL, five SALM, three NLRR, three Pal, two ISLR, three LRIG, two GPR, two Adlican, two Peroxidasin-like proteins, three Trk neurotrophin receptors, a yet unnamed protein AAI11068, and three AMIGO (Homma et al., 2008). The structures of LIG superfamily members are represented in *Figure 5.* Though their functions are possibly diverse, some LIG proteins have been shown to contribute in neuronal function and development. While neuronal activity may be a theme, an understanding of the cellular and CNS functions of the LIG family is still at its infancy. Major families of LIG protein (AMIGO, LINGO, NGL, NLRR, and LRIG) along with their recently discovered functions are reviewed below.

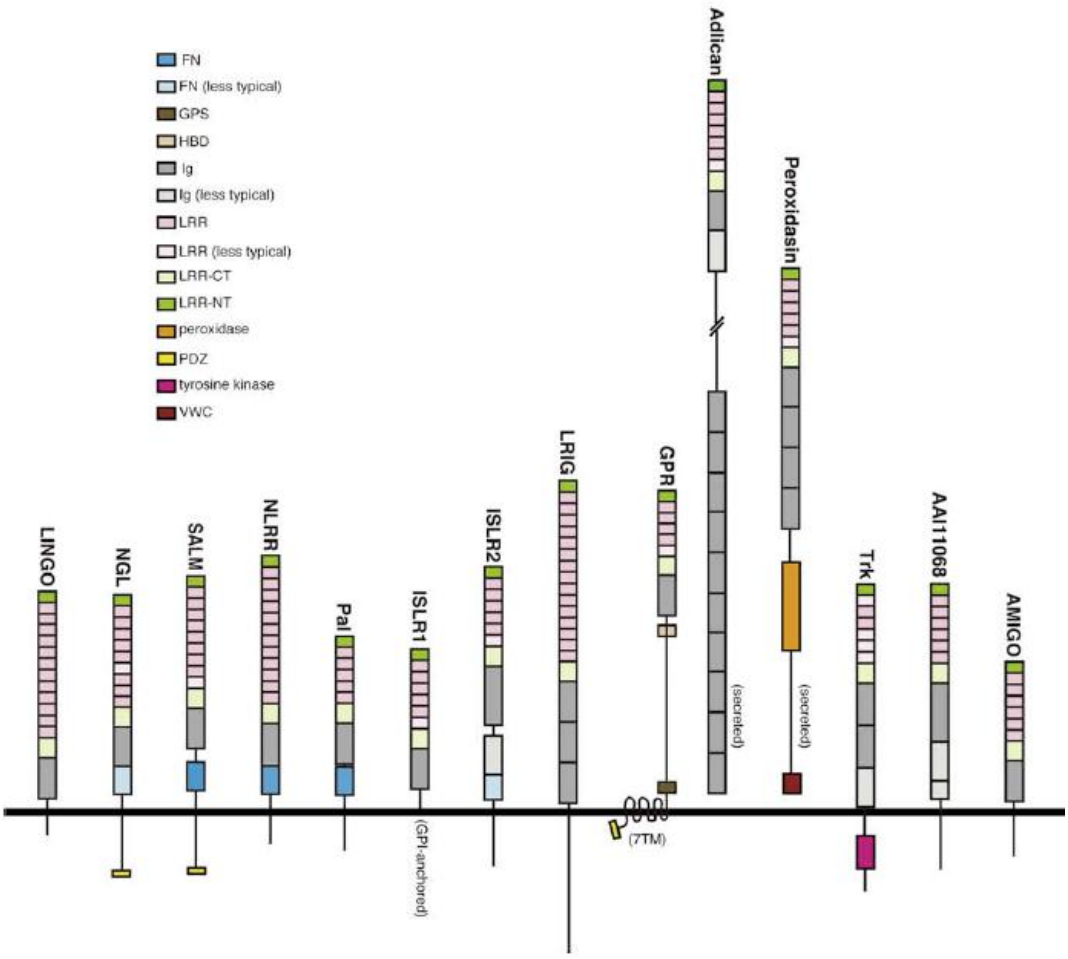

Figure 5: Summary of the structures of LIG superfamily's members (Homma et al., 2008)

Amphoterin-induced gene and ORF (AMIGO) Family: This family is named after a gene first found in rat hippocampal neurons. The family is consisted of three members: AMIGO-1, AMIGO-2, and AMIGO-3. They share the typical structure of type I transmembrane proteins with six LRRs and a single Immunoglobulin-like domain located next to the transmembrane region. Studies suggest exclusive expression of AMIGO in the nervous system and its significant contribution in hippocampal formation (Chen et al., 2005). Substrate-bound AMIGO ectodomain has been shown to promote neurite

extension of culture hippocampal neurons while the ectodomain itself, when added to the medium, restricts fasciculation of neurites. It is important to note that members of the AMIGO family demonstrate both homophilic and heterophilic binding activity, suggesting their role as novel cell adhesion molecules that monitor neuronal growth (Chen et al., 2005).

LRR and Ig domain containing Nogo Receptor interacting protein (LINGO) Family: LINGO-1 is the founding member of the LINGO family and was first discovered as the missing molecule required for NgR/p75 signaling, which directs inhibition of neuronal regeneration in spinal cord and brain injury (Chen et al., 2005). LINGO is a type I transmembrane protein with 12 LRRs and an Ig-like domain. LINGO 1 is specifically brain-enriched while the other three members of the family, LINGO 2-4, have wider distribution. Studies have indicated multiple functions of LINGO-1 in the Central Nervous System (CNS). Beside its mandatory role in neurite outgrowth inhibition, LINGO-1 contributes in modulating oligodendrocyte differentiation and myelination (Chen et al., 2005).

NGL-1: NGL-1 was discovered through interaction screen with Netrin G1, a highly conserved axonal guidance cue that monitors precise connections between neurons and their targets during development (Chen et al., 2005). NGL-1is exclusively enriched in brain and found abundantly in the striatum and the cerebral cortex. NGL-1 and Netrin G1 function together, playing an important role in the growth of thalamocortical neurons. As

a type I transmembrane protein NGL-1 has an extracellular domain containing nine LRRs and an Ig-like domain. The exact role of NGL-1 in the CNS remains to be investigated (Chen et al., 2005).

Neuronal Leucine-Rich Repeat (NLRR) Family: Four members of NLRR family have been identified and named NLRR1-4. All of them were shown to be expressed in brain; NLRR-2 and NLRR-3 are exclusively brain-enriched (Chen et al., 2005). The first three members of the mammalian NLRR are type I transmembrane protein with an extracellular domain consisted of 11-12 LRR repeats, an Ig-like domain, and a FN-III domain; NLRR-4 is unique within the family due to its lack of the Ig-like domain. The specific cellular and CNS functions of the NLRRs are still under investigation but they appear play an important role in neuronal function and development. NLRR-3 has been shown to be upregulated during cortical injury; NLRR-3 expression is involved in MAPK pathways signaling and is induced by EGF signaling. NLRR-4, on the other hand, plays a role in hippocampal dependent memory retention (Chen et al., 2005).

Leucine Rich Repeats and Immunoglobulin-like Domain (LRIG) Family: The LRIG family includes three members, LRIG-1, LRIG-2, and LRIG-3, whose structures consist of 15 LRRs and 3 Ig-like domains. LRIG-1 and LRIG-2 are expressed in various tissues, while LRIG-3 is principally expressed in non-neuronal tissues and in several exceptional sites within the nervous system (Homma et al., 2008). It has been suggested that LRIG is a regulator of stem cell quiescence, possibly through regulation of EGFR activity; LRIG-

1 maintains epidermal stem cells in a quiescent non-dividing state and a decrease in LRIG-1 expression may trigger proliferation (Jensen and Watt, 2006).

Epidermal Growth Factor Receptor Family and Cancer

Members of EGFR family have been shown to be involved in many types of cancer and many studies have illustrated and emphasized the extent and impact of EGFR overexpression in a variety of cancer types. EGFR is overexpressed on the cell surface of non-small cell lung cancer (NSCLC). Based on the new adenocarcinoma classification approved by the International Association for the Study of Lung Cancer, Korean researchers identified *EGFR* mutations in 50.5% of surgically resected lung adenocarcinomas in their centers. Use of EGFR tyrosine kinase inhibitors gefitinib and erlotinib has showed some effects on patients with adenocarcinomas identified as having activating EGFR mutations (Kobayashi et al**.,** 2005). ErbB2, also a member of EGFR family, is amplified in a 20-30% of breast cancers. As such, ErbB2-blocking antibodies such as Trastuzumab and Herceptin have been used in combination with chemotherapy in treatment of breast cancer (Harari and Yarden, 2000).

EGFR overexpression is also often found in prostate cancer, the most frequently diagnosed solid tumors in men. EGFR signaling pathway is suggested to help activate androgen receptor in androgen-deprived circumstances **(**Peraldo-Neia et al., 2011). In addition, approximately 50-60% of glioblastoma tumors have the EGFR overexpressed; the most common EGFR mutant, EGFRvIII, is expressed in 24-67% of cases (Heimberger et al., 2005). Inhibitory molecules and antibodies to EGFR are considered the most important drug candidates to target in prostate cancer and glioblastoma. Given

this data, the extent and impact of EFGR overexpression have been illustrated and emphasized in a variety of cancer types.

EGFR and LIG interactions

Given the role of EGFR signaling in cancer biology, the identification of Kek1 and LRIG1 as EGFR binding molecules, and the need for novel therapeutics, this project aimed to develop a simple and high throughput enzyme-linked-immunosorbent assay (ELISA) to screen for interactions between LIG molecules and members of the EGFR family, as well as among the LIG proteins themselves. It is expected that the assay could provide a quick screen to determine potential binding partners for more in-depth investigation.

Materials and Methods

Gateway Cloning System

The Gateway Cloning system allows DNA constructs to be cloned into a variety of vectors without acquiring the use of restriction enzyme. The technology is based primarily on the bacteriophage lambda site-specific recombination pathway. The gene of interest is integrated into the vectors at specific recombination sites called the *att* sites. The desired plasmids are obtained through BP and LR reactions, both of which are mediated by a mix of clonase system. The BP reaction directs recombination between a gene of interest flanked by two *attB* sites and a donor vector containing a ccdB gene flanked by two *attP* sites to produce an entry clone with the DNA fragment of interest flanked by two *attL* sites (B + P → L). The LR reaction then performs recombination

between the *attL* sites the donor vector and the *attR* sites of the destination vector to obtain the final expression clone with the gene of interest flanked by *attB* sites (L+R → B) Kanamycin and ampicillin resistance genes in the Gateway vectors, along with inclusion of a ccdB gene (toxic to *E. coli*) into the empty entry and destination vectors, provide important restrictions for successful selection of positive clones (Invitrogen, 2009).

Putative secreted Amigo1 (sA1) entry clones previously generated in the Duffy lab were used in an LR reaction with a pUAST-6XHIS/V5 destination vector to create an expression clone: 7μL pENTR sA1, 1μL of 150ng/μL pUAST-6XHIS/V5 and 2μL LR Clonase mix were incubated overnight. 5μL of the LR reaction were transformed into DH5α Max Cloning Efficiency cells and plated on LB agar plates containing 50μg/mL ampicillin. Colonies were grown overnight in small liquid LB cultures containing ampicillin and were then miniprepped using Qiagen's Qiaprep Spin Miniprep Kit. The sA1-6XHIS/V5 clones, along with previously generated constructs sLingo1-6XHIS/V5, sLingo1-GFP, sAmigo1-GFP, sAmigo2-6XHIS/V5, sAmigo3-6XHIS/V5, and sAmigo3-GFP, were midiprepped with Qiagen's Plasmid Midi Kit. The DNA constructs were then sent to sequencing at GENEWIZ, Inc. (Boston, MA) and the resulting chromatograms were analyzed using the Sequencher software. DNA concentrations were determined using NanoDrop Spectrophotometer.

Co-transfection

An S3 *Drosophila* cell line was maintained using Schneider's media with 12.5% FBS. Cells were seeded in a 6-well plate at $5x10^6$ cells/well in 2mL of media and transfected when the cells were 80-100% confluent using a Qiagen Effectene Transfection Kit. sDER-6XHIS/V5, sDER-GFP, sKEK1-6XHIS/V5, sKEK1-GFP, sKEK2-6XHIS/V5, sKEK2-GFP, sLINGO1-6XHIS/V5, sLINGO1-GFP, sAMIGO1-6XHIS/V5, sAMIGO1-GFP, sAMIGO2-6XHIS/V5, sAMIGO2-GFP, sAMIGO3-6XHIS/V5, sAMIGO3-GFP and ArmGAL4 (driver) were co-transfected into each seeded well, for a total of 800ng of DNA. The supernatants were harvested after one week and collected in Eppendorf tubes after running through a 22 um Whatman syringe filter.

Western Blots

Samples were run in the same pattern on two 8% SDS-PAGE gels, according to the protocol adapted by M. Arata and C. Ernst for the Duffy Lab. Proteins were transferred onto nitrocellulose membranes. One membrane was probed with polyclonal rabbit anti-GFP antibody diluted 1: 2000 with 5% NFDM in TBST, while the other was probed with monoclonal mouse anti-6XHIS/V5 antibody diluted 1:5000 with 5% NFDM in TBST. Goat-anti-mouse secondary antibody was subsequently used for membrane probed with monoclonal mouse anti-6XHIS/V5 antibody while goat-anti-rabbit secondary antibody was applied for membrane probed with polyclonal rabbit anti-GFP antibody. Both secondary antibodies were diluted 1:20,000 with 5% NFDM in TBST. The detection substrate used was a 1:1 peroxide: luminal solution, and blots were developed using an X-omat and Kodak film.

Quantification of 6XHis6XHIS/V5-tagged proteins

200 µl of 6XHIS/V5-tagged proteins in PBS/BSA were prepared at different concentrations, added to the wells of Qiagen Ni-NTA HisSorb Plates and incubated for 1 hour at room temperature. The wells were washed 3 times, 5 minutes/wash with PBS-Tween. After the PBS-Tween was removed and the wells were dry, 200 µl of primary monoclonal anti-6XHIS/V5 (diluted at 1: 5000 in PBS/BSA) was added and the plate was incubated overnight at 4°C. The next day the wells were washed 4 times, 10 minutes/wash with PBS-Tween. After the final wash, 200 µl of Goat-anti-mouse secondary antibody (diluted at 1: 20,000 in PBS/BSA) was added to each well and the plate was incubated at room temperature for 45-60 minutes. The final washes were performed 3 times-10 minute/wash before 200 µl of substrate solution TMB was added into the plate. Color development was monitored in a microplate reader at the wavelength of 650 nm.

Results

Generation of Secreted Isoforms

Cloning

Plasmids DNA of the following constructs were successfully transformed into *E. coli* cells, grown in liquid LB culture, and midi-prepped: sAMIGO1-GFP, sAMIGO2-6XHIS/V5, sLINGO1-6XHIS/V5, and sLINGO2-GFP. In contrast, growth was not observed on the plate and liquid cultures of *E. coli* cells transformed with sAMIGO1-

6XHIS/V5 and sAMIGO2-GFP constructs. So LR reactions (*Figure 6*) were attempted from pENTR sAMIGO1 and pUAST-6XHIS/V5 and pENTR sAMIGO2 and pUAST-GFP in order to generate new sAMIGO1-6XHIS/V5 and sAMIGO2-GFP constructs.

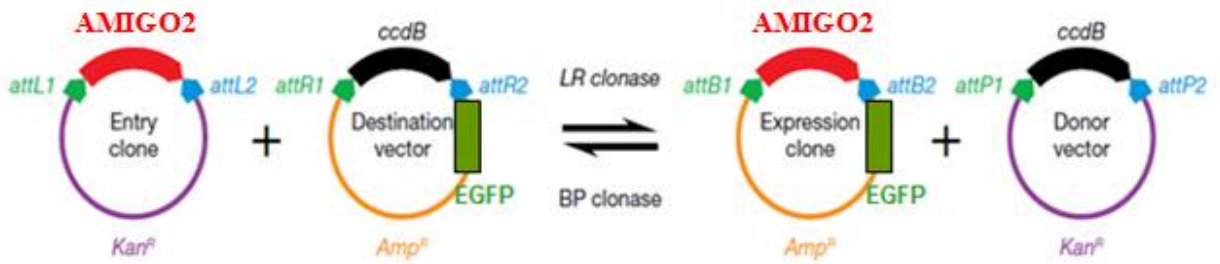

Figure 6: Gateway Cloning System (Invitrogen, 2009)

E. coli cells transformed with new sAMIGO1-6XHIS/V5 construct grew on Amp^r plates and in liquid culture, but colonies were not observed with cells transformed with new sAMIGO2-GFP construct despite multiple attempts. In total five constructs were purified and sent for sequencing for confirmation, sAMIGO1-6XHIS/V5, sAMIGO1-GFP, sAMIGO2-6XHIS/V5, sLINGO1-6XHIS/V5, and sLINGO2-GFP.

Sequence verification of Constructs

The sequencing results of the generated isoforms constructs were imported into Sequencher and contigs generated and experimental sequence was compared with the known sequence for each gene (*Figure 7*).

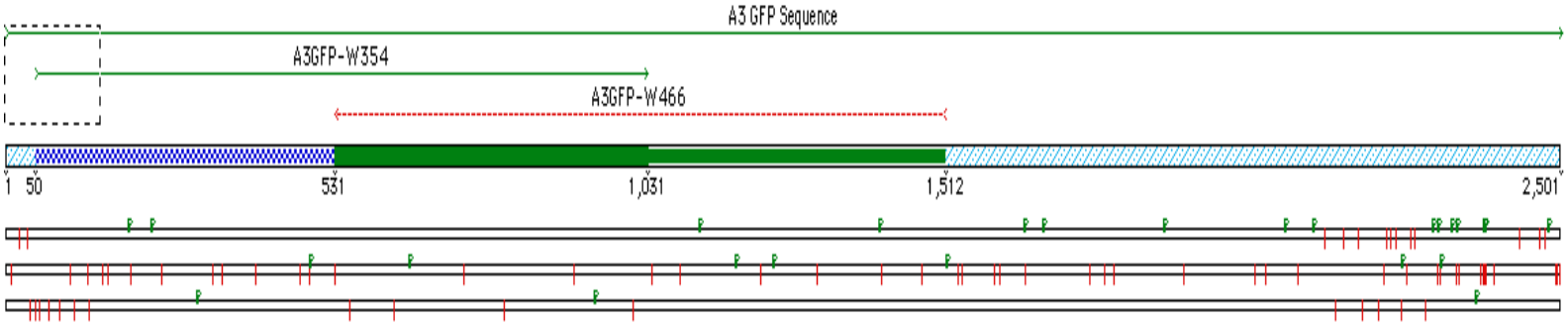

Figure 7: Contig Generation and Comparison between the Experimental and Experimental Sequences using Sequencer

The sequencing results were summarized in Table 1. The identities of the following constructs were verified: sLINGO1-6XHIS/V5, sAMIGO1-6XHIS/V5, sAMIGO2-6XHIS/V5, sAMIGO3-6XHIS/V5, and sAMIGO3-GFP.

Table 1: Verification of LIG plasmid DNA sequence by Sequencer Software

Sample Identity	Note	Verified Construct
sLINGO1-6XHIS/V5	Point mutation at 5' polylinker region	Yes
sLINGO1-GFP	No priming due to low DNA concentration	No
sAMIGO1-6XHIS/V5	One missing nucleotide at 5' polylinker region	Yes
sAMIGO1-GFP	One insertion right after the start codon	No
sAMIGO2-6XHIS/V5	100% consistent with the theoretical sequence	Yes
sAMIGO3-6XHIS/V5	One missing nucleotide at 5' polylinker region and one silent mutation in the coding region (GAA observed instead of GAG but both coded for Glutamic Acid)	Yes
sAMIGO3-GFP	Point mutation in polylinker region, 5'AttB site, 3'AttB site, and silent mutation in the coding region (GAA instead of GAG but both coded for Glutamic Acid)	Yes

It should be noted from Table 1 that sAMIGO1-GFP construct could not be used for protein expression due to the insertion of an additional nucleotide right after the start codon followed by severe frameshift mutation. The construct sLINGO1-GFP could not be sequenced due to "No priming" error which could possibly be attributed to poor quality of the DNA template or low DNA concentration. Though mutations were also

reported in the other constructs shown in Table 1, they do not result in translation of erroneous protein sequences; therefore, sLINGO1-6XHIS/V5, sAMIGO1-6XHIS/V5, sAMIGO2-6XHIS/V5, sAMIGO3-6XHIS/V5, and sAMIGO3-GFP could be used for protein expression. Together with the isoforms of sKEK1-6XHIS/V5, sKEK1-GFP, sKEK2-6XHIS/V5, sKEK2-GFP, sdEGFR-6XHIS/V5, and sdEGFR-GFP, which were previously generated and verified in the lab, a total of 11 clones of secreted isoforms of LIG proteins and dEGFR (Table 2) were prepared.

Table 2: Summary of Secreted Tagged Isoforms Verified by Sequencing

Construct	DNA concentration (ug/ul)
sdEGFR-6XHIS/V5	3.2
sKEK1-6XHIS/V5	2.8
sKEK2-6XHIS/V5	2.2
sLINGO1-6XHIS/V5	0.8
sAMIGO1-6XHIS/V5	1.15
sAMIGO2-6XHIS/V5	1.36
sAMIGO3-6XHIS/V5	1.26
sdEGFR-GFP	2.8
sKek1-GFP	5.3
sKek2-GFP	1.3
sAMIGO3-GFP	1.5

Protein Expression, Verification and Quantification

To determine if constructs were able to produce the predicted secreted molecules the *Drosophila* GAL4/UAS cell culture expression system was used (Klueg et al., 2002).

In this system both the expression clone (e.g. sdEGFR-GFP) and the inducer clone (ArmGAL4) were co-transfected into *Drosophila* S3 cells (*Figure 8*).

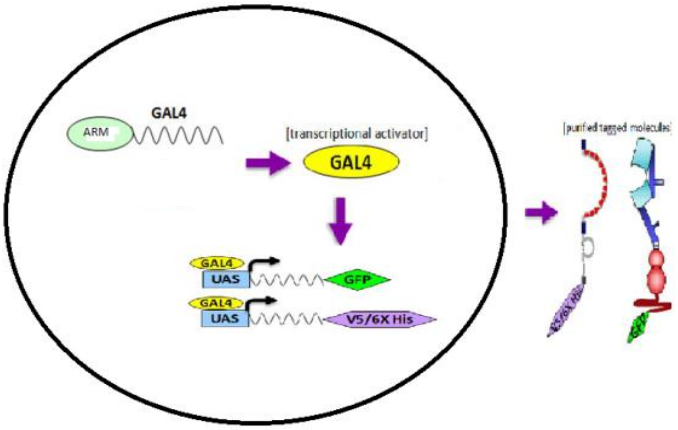

Figure 8: Drosophila cell expression

Western Blot Verification for GFP-tagged Proteins

After one week of transfection, supernatants were collected, separated from the cells, and probed for secreted proteins by Western Blot. The GFP-tagged proteins were probed with αGFP antibody is shown in Figure 9.

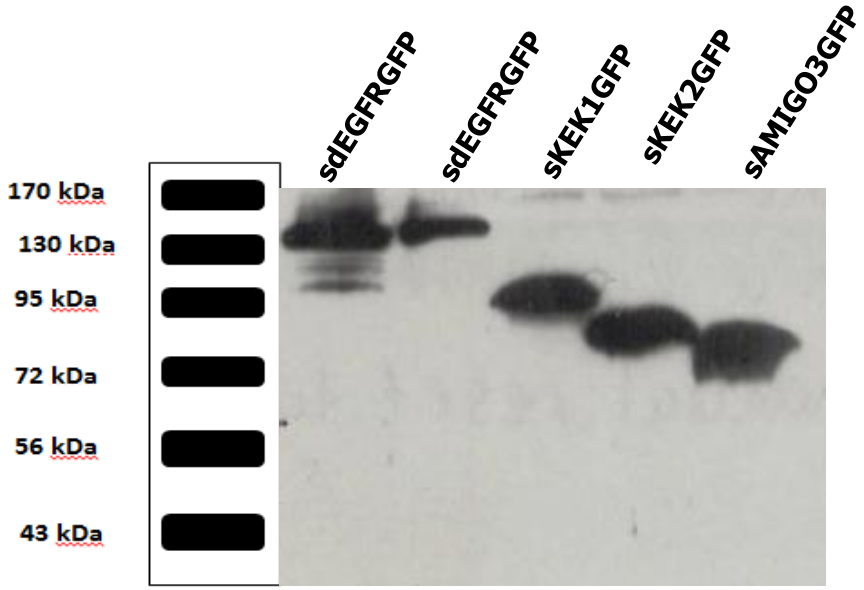

Figure 9: Western Blot Verification of EGFP-tagged proteins

For sdEGFR-GFP, sKEK1-GFP, sKEK2-GFP, and sAMIGO3-GFP single bands were detected at approximately the correct sizes. The theoretical sizes and experimental sizes of the EGFP-tagged proteins were compared in Table 3.

Table 3: Comparison between theoretical and experimental sizes GFP-tagged protein

GFP-tagged Protein	Theoretical Protein Size (kDa)	Experimentally Determined Protein Size (kDa)
sdEGFR-GFP	126.91	130
sKEK1-GFP	78.82	93
sKEK2-GFP	72.35	80
sAMIGO3-GFP	71.35	75

Based on the presence of major single bands for each protein (*Figure 9*) and their relative sizes, secreted versions of sdEGFR-GFP, sKEK1-GFP, sKEK2-GFP, and sAMIGO3-GFP are produced and appear stable using this system.

Western Blot and ELISA Verification for 6XHIS/V5-tagged Proteins

As done for the GFP tagged secreted molecules, the expression of 6XHIS/V5-tagged proteins was also assessed. In this case they could be verified both by Western blot and ELISA due to the ability of the 6XHIS tag to bind to a Ni-NTA-coated surface (*Figure 10*).

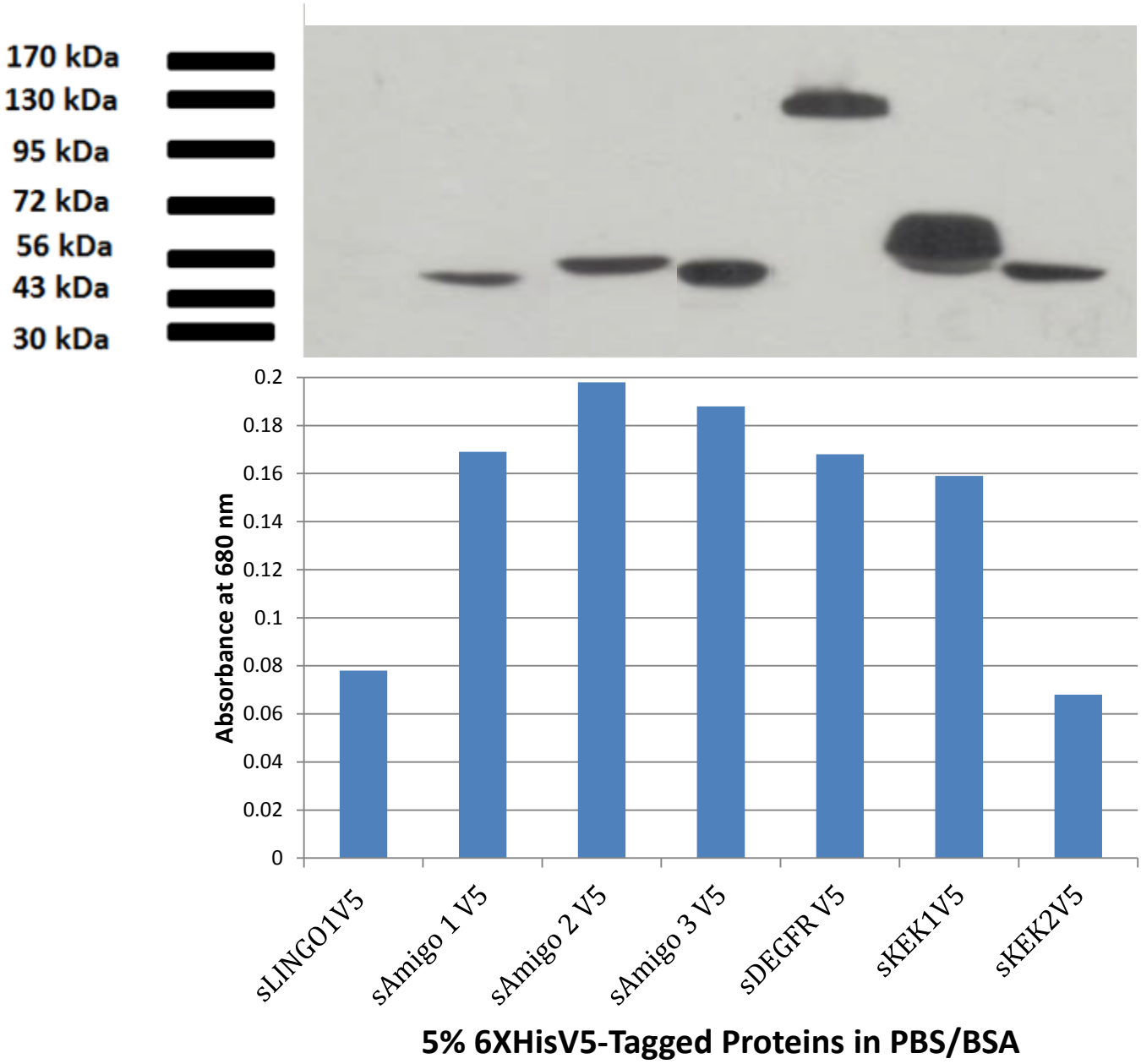

Figure 10: Western Blot and ELISA Verification of 6XHisV5-Tagged Proteins

The Western blot and ELISA results were mostly consistent. The presence of the protein on the blot as represented by a band generally corresponded to the high A_{680} readings in the ELISA assay as demonstrated in cases of sAMIGO1-6XHIS/V5, sAMIGO2-6XHIS/V5, sAMIGO3-6XHIS/V5, sdEGFR-6XHIS/V5, and sKEK1-6XHIS/V5. In contrast, sLINGO1-6XHIS/V5 was not detected by the Western blot and its optical density reading from ELISA was relatively low. It should be noted that the cloning and protein expression for sLINGO1 constructs, tagged with either 6XHIS/V5 or GFP, met with no real success. The sLINGO1-GFP plasmid constructs could not be cloned properly, while sLINGO1-6XHIS/V5 gave no expression even its sequence appears correct. The only discrepancy in comparison between the Western and ELISA assays was sKEK2-6XHIS/V5, which gave band on the Western blot, but low absorbance reading with ELISA. Many factors could explain the difference: the difference in sensitivity of the two assays, lack of standard for a baseline in ELISA, or the mistaken sample loading. Theoretical and experimental sizes of the 6XHis-tagged proteins were compared in Table 4.

Table 4: Comparison between theoretical and experimental sizes of 6XHIS/V5-tagged proteins.

6XHIS/V5-tagged Protein	Theoretical Protein Size (kDa)	Experimentally Determined Protein Size (kDa)
sAMIGO1-6XHIS/V5	45.87	50
sAMIGO2-6XHIS/V5	48.47	53
sAMIGO3-6XHIS/V5	45.92	48
sdEGFR-6XHIS/V5	52.81	56
sKEK1-6XHIS/V5	46.59	48
sKEK2-6XHIS/V5	100.9	120

Table 4 demonstrated that the difference between theoretical and experimentally determined size of 6XHIS/V5-tagged secreted proteins were minimal. Size verification on Table 4 and Western Blot and ELISA shown in Figure 10 supported the presence of sAMIGO1-6XHIS/V5, sAMIGO2-6XHIS/V5, sAMIGO3-6XHIS/V5, sdEGFR-6XHIS/V5, and sKEK1-6XHIS/V5 in the harvested supernatants.

Feasibility of High Throughput ELISA Interaction Assay

With the knowledge that secreted versions of LIGs and the receptor could be generated a preliminary test was run to assess the feasibility of an ELISA based interaction assay in a 96 well plate format (Figure 11).

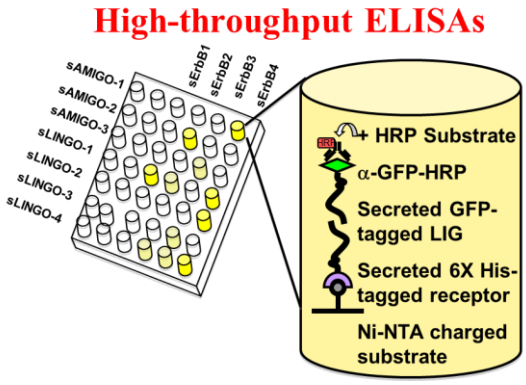

Figure 8: Schematic Interaction Assay

For this, the ability of Kek1 to interact with the dEGFR was assessed relative to cell supernatant lacking Kek1 expression (Figure 12). Although preliminary, a stronger signal was detected when dEGFR-expressing supernatant was incubated with Kek1-expressing supernatant as compared to supernatant from control cells lacking Kek1 expression.

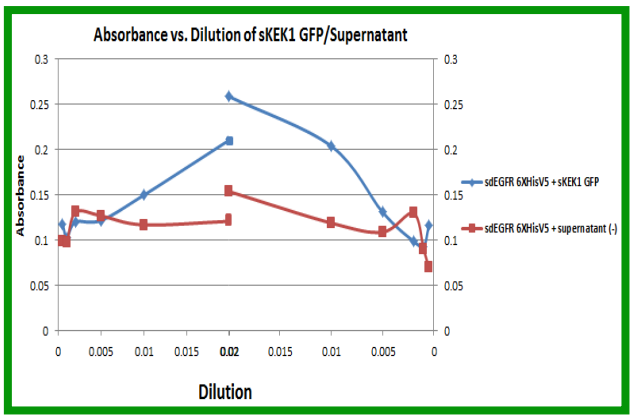

Figure 12: ELISA Based Interaction Assay

Discussion

The study indicated that tagged and secreted versions of dEGFR, Kek1, Kek2, and Amigo 1-3 have been successfully generated. Discrete bands shown on the Western blots (*Figure 9 and 10*) suggested that full-size proteins of LIG family and dEGFR have been produced and not subjected to major degradation or processing steps. Using the *Drosophila* cell culture expression system appeared to work effectively as it produced stable secreted versions of the proteins. The only exception was LINGO1 constructs for which I was unable to generate the GFP tagged clone or demonstrable expression of the 6XHIS/V5 tagged protein by Western Blot. As the sequence of LINGO1-6XHIS/V5 was wild type, it will be important to clarify if lack of expression of the LINGO1-6XHIS/V5 was due to poor transfection or lack of protein stability.

Initial data also suggested that it was feasible to assess concentrations of tagged molecules via ELISA and Fluorescence Spectrometry. Differences were observed in the readings of tagged proteins and negative control. In the next step of the study, a standardized approach for quantification of both V5 and GFP-tagged proteins should be developed. Parameters of the plate reader could be better defined to give reproducible analyses of levels of GFP tagged molecules. This is critical since it is necessary to define the input levels of each protein for the ELISA interaction assay.

Primary tests showed the potential of the interaction assay to evaluate the level of communication between the members of the LIG superfamily and dEGFR. Testing supernatants expressing secreted forms of dEGFR and KEK1 in the ELISA interaction

assay resulted in a higher signal than was recorded for the interaction between dEGFR and supernatant only. In future studies, the conditions for the ELISA interaction assay, such as incubation time and wash time, should be optimized to allow for easy distinction between high affinity and low affinity interactions. The development of the assay shows great promise as screening test for more in-depth investigations of the LIG proteins and their ability to interact with members of the EGFR and other receptor families. Ultimately this will open the path for more insight on this superfamily for which little functional data exists.

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