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Swapping Stories:
Gaining insight to cancer therapeutics and EGFR signaling from flies

A Major Qualifying Project Report

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ABSTRACT

Breast cancer affects ~1 million new women yearly. Overexpression of ErbB2, a member of the EGFR family, is a causative factor in 30% of these cases and is targeted by the cancer drug Herceptin. Strikingly, studies of ErbB2 and the *Drosophila* EGFR have uncovered a close structural relationship. Using domain swaps between dEGFR and ErbB2, I have been investigating inhibition of dEGFR by the transmembrane molecule Kekkon1 with the hope that such insight may lead to novel cancer drugs.

ACKNOWLEDGEMENTS

I would like to thank Duff first and foremost, for being a remarkable advisor, PI, and personal mentor. You helped me make wise choices throughout my time as a WPI undergraduate and I hope you will continue to stay involved throughout the future. Most importantly, you motivated and pushed me to work harder than I would have dreamed possible.

I would also like to extend an enormous thank you to all of the Duffy lab members, past and present. Christina Ernst, Aditi Gautam, Prachi Gupta, Harita Haridas, Edie Plada: you ladies taught me the basics when I was starting out and have continually aided me throughout my time in the lab, for which I am forever grateful. I also want to thank Michelle Arata for her infinite help with cell culture and Western Blots. The Duffy lab has truly been a wonderful environment in which to learn, grow, and sometimes indulge in silly antics. I have looked forward to lab every single day, and I know that you girls have been an integral part of that. The Duffy lab was more than just a lab in which to do my MQP - it was here that my love for science grew and my friendships blossomed.

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To Liz Ryder, thank you for the use of the microscope, reagents and gel equipment. These contributions allowed me to gain more understanding of my project. Additional thanks to Molly McShea for running our very first Western Blots together.

Lastly, I would not be here without the help of the Biology and Biotechnology Department at WPI. I have enjoyed interacting with the faculty and have grown incredibly through the classes I have taken as an undergraduate. I am walking away from this institution prepared and eager to succeed.

I am eternally grateful to WPI, Duff and the Duffy lab for providing me with the knowledge and experience I need to pursue my dreams. I would not be the scientist I am today without the guidance and dedication from everyone.

TABLE OF CONTENTS

INTRODUCTION

Cancer encompasses an entire group of diseases characterized by uncontrolled growth and spread of abnormal cells. In the year 2009, it was estimated that close to 1.5 million new cancer cases were diagnosed with ~ 600,000 cancer-related deaths that year (American Cancer Society, 2009). The causes of cancer are as varied as the tissues in which it can develop. It can be the result of external factors, such as tobacco use, infectious organisms, and radiation, as well as of internal factors, like inherited mutations, hormones, and immune conditions. Sometimes, many years may pass between exposure to external factors and detectable cancer in a patient. Current treatments include surgery, chemotherapy, hormone therapy, biological therapy, and targeted therapy. As technology and science evolve, so does our knowledge of the molecular mechanisms underlying cancer. A 2008 report from the National Cancer Institute shows in fact that for the first time since the report was first issued in 1998, both incidence and death rates for all cancers combined have decreased for both men and women. Through ongoing research efforts to understand the molecular pathways dysregulated by cancerous cells, it may one day be possible to create specific targeting therapies to prevent this dysregulation and thereby inhibit cancer progression at the cellular level.

Breast Cancer

Of the new cases of cancer diagnosed in 2009, almost 30%, or nearly 190,000 new cases were breast cancer cases (American Cancer Society, 2009). Breast cancer is the most frequently diagnosed cancer in women, causing an estimated 40,000 female deaths in 2009. Various factors can predispose a female for breast cancer, with age predominating. Certain external factors include being overweight or obese after menopause, use of menopausal hormone therapies, physical inactivity, and consumption of more than one alcoholic beverage per day. Additionally, risk is increased by family history of breast cancer and inherited genetic mutations in the breast cancer susceptibility genes BRCA1 and BRCA2.

The treatment of breast cancer varies from case to case, but most often includes surgery to remove the cancer from the breast and to determine the severity of the disease. Surgical removal is often combined with radiation therapy, chemotherapy, hormone therapy, and/or biologic therapy. Radiation destroys any cancer cells remaining in the breast, chest wall, or underarm area after surgery, and even serves to reduce tumor size *prior* to surgery. Systemic therapies employ anti-cancer drugs that travel through the bloodstream to all the parts of the body, and include chemotherapy, hormone therapy, and biologic therapy (American Cancer Society, 2009).

It has been found that 15-30% of breast cancers overproduce HER2/ ErbB2, a growth promoting protein (Society, 2009). Additionally, these types of cancer tend to be aggressive forms of breast cancer, growing faster and with a higher recurrence rate. These ErbB2 dependent cancers are currently treated with Herceptin (tratuzumab), a monoclonal antibody that targets the ErbB2 protein (Shepard et al., 2008). Studies have shown that treatment with Herceptin in early stage breast cancer combined with standard chemotherapy reduces the risk of recurrence and death by anywhere from 33-52% compared to chemotherapy alone (American Cancer Society, 2009).

Epidermal Growth Factor Receptor (EGFR) Family

The ErbB2 protein is one of the four receptors (ErbB1-ErbB4) belonging to the epidermal growth factor receptor family in vertebrates, a family of receptor tyrosine kinases named after the originally discovered receptor ErbB1 (EGFR). Homologs of EGFR can be found in a wide range of organisms, underlying its significance as a central player in cell-cell communication or signal transduction. These receptors, along with their associated ligands, have been implicated in a wide array of cellular behaviors including, cellular growth, cell fate determination, differentiation, migration, as well as normal tissue turnover and wound healing (Bazley and Gullick, 2005).

Members of the epidermal growth factor receptor family each have a conserved protein tyrosine kinase domain in the cytoplasm, a transmembrane domain, and a glycosylated, extracellular ligand-binding domain consisting of four domains, labeled I-IV (Burgess et al., 2003). Domains I and III collectively form the ligand-binding site, while

domains II and IV are homologous, cysteine-rich regions. ErbB1, ErbB3, and ErbB4 are normally found in a bent conformation when inactive, due to tethering interactions between domains II and IV. Ligand binding pulls domains I and III together, locking the receptor open in an active conformation (Schlessinger, 2002). The dimerization arm in domain II is then exposed, promoting receptor dimerization (Figure 1). One receptor monomer phosphorylates the other monomer, initiating downstream intracellular signaling events that control cell migration, proliferation, adhesion, and differentiation.

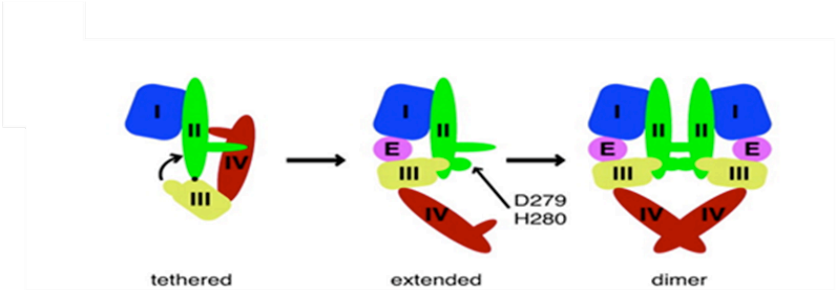

Figure 1. A structural model for EGFR activation (refer to text for details)(Burgess et al., 2003)

EGFR dimerization and activation often is accompanied by a mitogen-activated protein (MAP) kinase signaling pathway composed of three enzymes (MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase) that are activated sequentially. The MAP kinase phosphorylation cascade occurs within the cytosol, eventually leading to phosphorylation of various transcription factors, which alter the transcriptional profile of the cell(Figure 2). Upon ligand binding and receptor autophosphorylation, binding of a adaptor molecules, like growth factor receptor bound protein 2 (GRB2) occurs (Kolch et al., 2002). This is mediated by the recognition of phosphotyrosines on EGFR via phosphotyrosine binding motifs, such as GRB2's SH2 domain. GRB2 comes to the cell membrane bound to SOS, a guanine nucleotide exchange factor (GEF) protein that in turn activates the small G-protein Ras by enhancing the exchange of guanosine diphosphate (GDP) for a guanosine triphosphate (GTP). This activation changes the physical conformation of Ras, allowing it to bind the effector protein Raf-1, enabling *its* recruitment to the cell membrane also. Raf-1 is a serine/threonine (S/T) kinase and proceeds to

phosphorylate and activate MEK, a MAPK/ERK dual specificity kinase (S/T and tyrosine), which then phosphorylates and activates extracellular-signal-regulated kinase (ERK). At this point, many substrates are available in the cystosol for ERK or MAPK, in addition to the nucleus, to where the kinase can translocate to and phosphorylate nuclear transcription factors directly. In addition to the removal of ligand there are a wide array of mechanisms for subsequently halting or downregulating the RTK response, including engulfment of the ligand/receptor complex by receptor-mediated endocytosis (Duffy and Perrimon, 1994).

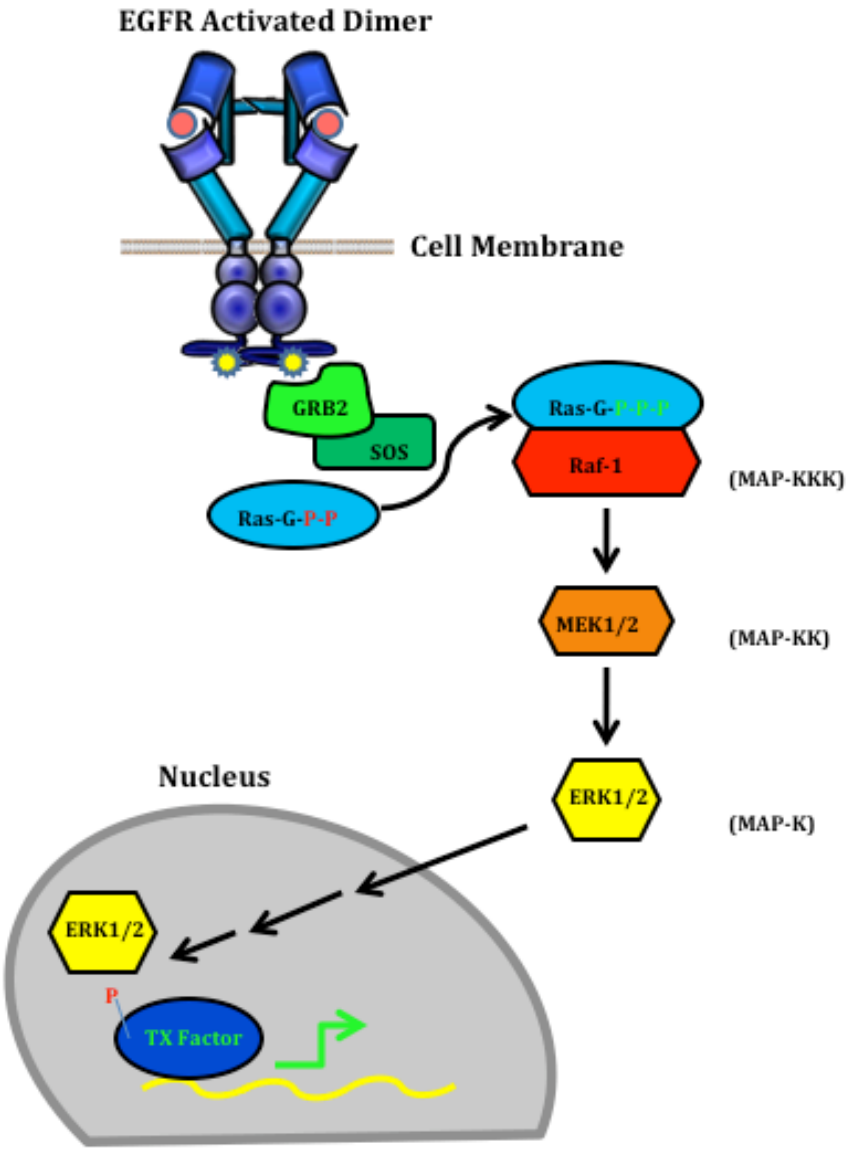

Figure 2. ErbB1 activated MAP kinase signaling cascade A structural model for EGFR activation (refer to text for details)

ErbB2

The second member of the EGFR family, ErbB2, is distinct from the rest of the family in that there is no known soluble ligand that directly interacts with the receptor, and it is the only family member is capable of transforming cells when it is overexpressed (Alvarado et al., 2009). The recently solved crystal structure of ErbB2 by Alvarado et al., suggests that in fact ErbB2 is the closest structural relative of the *Drosophila melanogaster* EGFR homolog, dEGFR/DER, sharing a 35% sequence identity over domain I-IV (Figure 3R). Both of these receptors lack the intramolecular tether between domains II and IV that hold ErbB1, ErbB3, and ErbB4 in a closed conformation, and thus maintain an open conformation (Figure 3L). Contrary to what was once believed, it now appears that this open conformation does not imply constitutive activation, as autoinhibition is provided by unique interdomain interactions seen in both ErbB2 and DER (Alvarado et al., 2009)

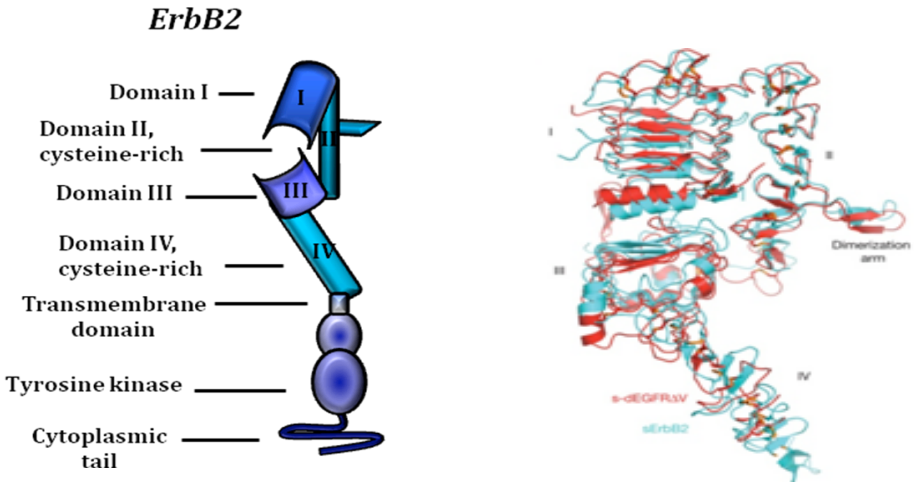

Figure 3. (L) ErbB2 structure

(R) Overlaid crystal structures of secreted DER (red) and secreted ErbB2 (blue), (Alvarado et al., 2009)

Domain II of ErbB2 appears to be in an 'inactive' conformation, lacking a 12 degree bend between two of its modules that is necessary for ligand-induced dimerization. This

inactive-like domain II is detectable in bent unliganded ErbB3 and ErbB4, as well as in DER. Additionally, direct interactions between domains I and III are evident in both ErbB2 and DER. These interactions aid in maintaining the receptors stable in their open conformation. The domain I/III interactions also have an autoinhibitory effect by forcing the two parts of the ligand-binding site close to each other, preventing ligand from binding. A consequence of this interaction is another interaction between domains I and II, which further stabilizes the inactive domain II. These contacts are conserved in ErbB2, DER, as well as in unliganded ErbB3 and ErbB4 (Alvarado et al., 2009)

Oogenesis in Drosophila melanogaster

Drosophila flies undergo meroistic oogenesis, development of an egg in which cytoplasmic connections remain between the cells produced. Each oogonium divides four times to produce a clone of interconnected 16 cells known as cystocytes. Only one of the 16 cystosytes can go on to become an ovum, while all the rest become nurse cells. These nurse cells (Figure 4) provide and store nutrients in the egg yolk that an eventual embryo will require (Bastock and St Johnston, 2008).

The maturing, pre-fertilized egg is encased by follicle cells (Figure 4), which allow for the exchange of information between them and the oocyte. The follicular cells originate from a second population of stem cells of somatic origin, and form the monolayered epithelium which covers each germline cyst (Lin and Spradling, 1993).

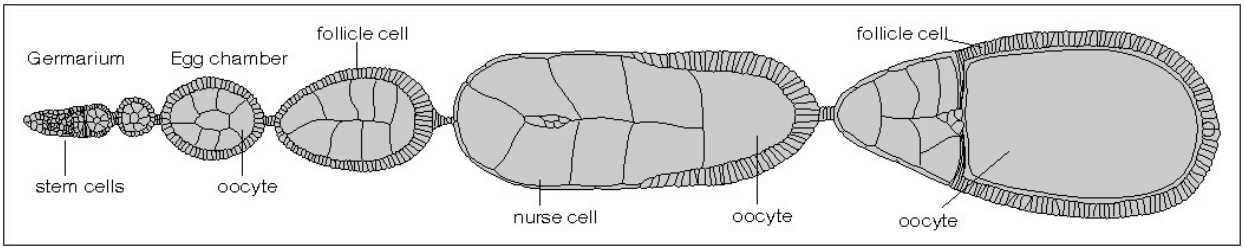

Figure 4. Stages of *Drosophila* oogenesis

(University of Newfoundland, 2010)

Formation of the embryonic body pattern is dependent on EGFR signaling and requires communication between the oocyte and its overlying follicle cells (Nilson and Schupbach, 1999). Localized activation of DER differentiates a particular subpopulation of follicle cells, and establishes this crucial symmetry in the follicular epithelium. In the beginning of oogenesis, DER activation establishes anterioposterior polarity of the egg chamber, while in the middle of oogenesis, the confinement of DER activity to the dorsal follicle cells establishes dorsoventral polarity. Specifically, DER activity in the follicular epithelium of the ovary is controlled by the localization of its ligand Gurken, which acts as the guidance cue. This role for DER in development is independent of the Raf-MAP kinase signaling pathway (Duchek and Rorth, 2001; Nilson and Schupbach, 1999).

Eye Structure and Development in Drosophila melanogaster

The adult Drosophila eye is composed of nearly 800 hexagonal ommatidia, along with sensory hairs that project from the surface. Each ommatidium is composed of eight photoreceptor cells (Figure 5), denoted R1-R8, each of which is surrounded by a rod-like element containing photoreceptor elements known as a rhabdomere. R1-R6 are located radially around R7 and R8. Each ommatidium is also surrounded by two primary pigment cells, which are further surrounded by six secondary pigment cells. These are shared by adjacent ommatidia. In total, each ommatidia contains 22 cells, from which a cluster of eight axons then run posteriorly into a pre-optic stalk (Freeman, 1997).

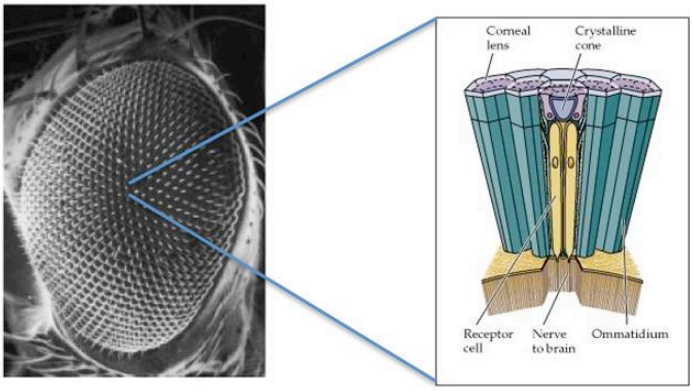

Figure 5. Structure of the *Drosophila* eye and ommatidia (NCBI, http://www.ncbi.nlm.nih.gov/bookshelf/picrender.fcgi?book=neurosci&part=A1498&blobname=ch22f10.jpg**)**

Cells in the eye are determined by inductive signaling. Specifically, DER signaling, activated by the Spitz ligand, is used repeatedly to trigger the differentiation of each of the cell types. Studies have shown that Keren ligand acts in cooperation with Spitz to control photoreceptor R8 spacing, cell clustering and survival (Brown et al., 2007). The DER signaling cascade first recruits photoreceptors, followed by cone receptors, and lastly pigment cells.

DER and Kekkon 1

As noted above, DER is very similar in structure to the human EGFRs. Domains I-IV are conserved in DER, with the additional presence of a cysteine-rich domain (V) at the end of the extracellular portion of the molecule (Figure 4). Functionally, DER is required for various developmental processes through the *Drosophila* life cycle. In oogenesis, activation of the EGFR pathway in follicle cells establishes both anteroposterior and dorsoventral fates. During embryogenesis, it establishes ventral cell fates, determination of segmental identity, maintenance of amnioserosa and ventral neuroectodermal cells, germband retraction, cell fate specification in the central nervous system, and production of cuticle. In larval development, EGFR is involved in cell proliferation, vein formation, and eye development (Alvarado et al., 2004).

A *Drosophila* transmembrane molecule, Kekkon 1, was identified as an inhibitor of DER, acting in a negative feedback loop to regulate the activity of the DER tyrosine kinase. Kekkon 1 contains leucine-rich repeats (LRR) and an immunoglobulin (Ig) domain (Figure 6), making it the founding member of a LIG family of six related transmembrane proteins in *Drosophila* that contain LRRs and a single Ig domain in their extracellular regions (Duffy 3). Biochemically, co-immunoprecipitation experiments have shown that Kek1 physically binds to DER. When Kek1 is overexpressed in oogenesis *in vivo* using the UAS GAL4 system, a ventralized eggshell phenotype is obtained. This is manifested as longer eggs with a reduction or complete loss of dorsal appendages. Loss-of-function DER studies yield similar results, consistent with the ability of Kek1 to inhibit the receptor. Additional loss-of-function studies have indicated that Kek 1 is a general inhibitor of DER throughout development (Alvarado et al., 2004).

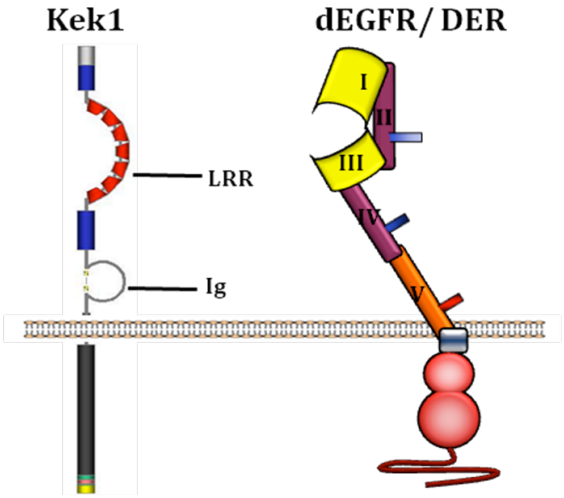

Figure 6. Kek1 (left) and DER (right) structures. (refer to text for details)

Previous work with Kek1 and DER

Using various chimeric Kekkon constructs, it has been determined that the extracellular and transmembrane portions of Kek1 are sufficient to direct interaction with DER *in vivo* in a ligand-independent manner. Kek1 mediated inhibition of DER signaling is believed to be a bipartite process, with the LRRs dictating DER binding and the juxta/transmembrane region contributing to the inhibition. To better understand the physical interactions leading to this binding, a screen was carried out to identify mutations in DER that suppress Kek1 binding. Five mutations were recovered and named SOKs, for suppressor of Kek1 (Alvarado et al., 2004). These dominant suppressors, as the name suggests, prevented Kek1's inhibition of DER. Interestingly, the SOKs all mapped to the cysteine-rich domain V of DER. Based on this data, it appears that domain V is a key player in DER's interaction with Kek1 and subsequent inhibition. Accordingly, co-immunoprecipitation data obtained in the Duffy lab shows that a truncated DER lacking domain V shows a decreased affinity in binding Kek1 *in vitro*. The addition of domain V to ErbB1 does not confer binding ability, suggesting that domain V is not sufficient for this interaction. However, recent structural data implies that ErbB1 and DER may not be similar enough for such domain swaps, as the chimeric protein is unlikely to maintain a relevant shape.

To better understand the interaction of Kek1 and DER, I instead adopted the use of ErbB2 rather than ErbB1 for domain swaps. Given the structural similarity recent reported between DER and ErbB2, by using ErbB2 rather than ErbB1 I hoped to better maintain the structural integrity of the subsequent protein chimeras for my domain mapping experiments. *In vivo* analyses of one of these successfully generated chimeric ErbB2/DER constructs, $DER^{SI\text{-}Iva}$, shows that the protein chimera retains its activity and localization. This validated my approach, confirming it will ultimately be a fruitful method for dissecting sequence elements in the receptor involved in the Kek1/DER interaction. Thus, this scheme of creating ErbB2/DER chimeric constructs, followed by *in vivo* and *in vitro* testing, establishes a system in which to better understand the necessity/sufficiency of DER's

domains in binding Kek1. Such insights are essential for long-term goals involving the development of novel Kek1 based therapeutics that will specifically target, bind, and inhibit members of the human ErbB family, thus providing a novel new way to treat ErbB-dependent cancers.

MATERIALS AND METHODS

Generation of DNA Constructs

Part 1: Creating Fragments by PCR

All constructs were generated from either pENTR ErbB2 or pENTR WT-DER, full length constructs in a Gateway cloning-adapted pENTR vector. All PCR reactions were run in 50 µl, using Vent and 10x polymerase buffer (NEB) and supplying with $MgSO_4$. 5' and 3' primers were designed for each fragment, and 5µl were used for each at a concentration of 200ng/µl. Enough template DNA was added to yield 100-150ng. 3 µl of 2.5mM dNTPs were supplied for each reaction. The conditions for PCR were determined based on the Tm's of the primers and the length of the individual fragments. Reactions were normally run at an annealing temperature of 60 degrees C, and an extension time of 2min/kb for 35 cycles. Fragments were checked on a 0.8% agarose gel and band-purified using Qiagens Qiaquick Gel Extraction kit.

Part 2: Stitching PCR Fragments to Yield Full Length Constructs

Each fragment was designed to have an overhanging sequence complementary to the adjoining fragment. The overhangs from each fragment bond with one another during the first round of the stitching reaction, and are extended by polymerase to form the final template (Figure 7). This then serves as the template for the subsequent PCR rounds, as for a normal PCR reaction.

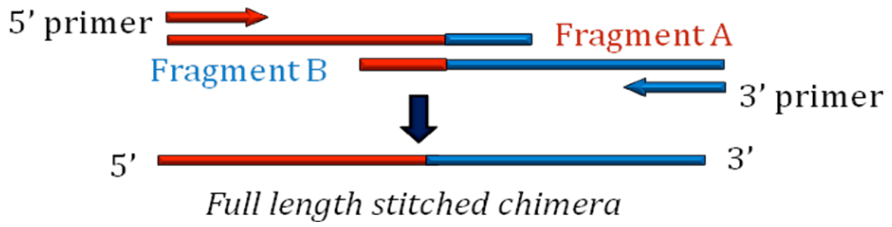

Figure 7. Stitching PCR schematic

All stitching PCR reactions were carried out in 50 µl, using Phusion, 10X polymerase buffer (NEB) and supplying with DMSO. 5' and 3' end primers were designed for each fragment, and 5µl were used for each at a concentration of 200ng/µl. The two previous fragments were used as template DNA, and about 150ng of each were added. 3 µl of 2.5mM

dNTPs were supplied for each reaction. The conditions for PCR were determined based on the Tm's of the primers and the length of the individual fragments. Phusion enzyme is activate at higher temperatures than Vent, so the reactions were normally run at an annealing temperature of 68º C, and an extension time of 1min/kb for 35 cycles. Full length constructs were checked on a 0.8% agarose gel and band-purified using Qiagens Qiaquick Gel Extraction kit.

Part 3: Gateway Cloning System

The Gateway cloning system was used to shuttle the chimeric genes into the final expression vector. The *"Gateway Cloning System" (*adapted from Premier Biosoft International's "Gateway® Cloning") is based on the lambda phage site-specific recombination pathway between the att sites of the bacterial as well as the phage DNA. Recombination is made possible by the phage-encoded integrase protein and the host-encoded accessory protein integration host factor. BP and LR reactions, tailored from this natural process, are the basis of the Gateway system and allow for gene sequences to be easily shuttled between vectors without the use of restriction enzymes.

The BP reaction occurs between the gene of interest flanked by attB sites, in this case the stitched PCR product, and the donor vector flanked by attP sites. Recombination occurs between these sites and the result is an entry clone with the gene of interest flanked by attL sites. The LR reaction is then a recombination between the attL and attR sites of the entry clone and a destination vector flanked by attR sites. The result is an expression clone containing the sequence of interest flanked by attB sites that can be expressed in cell culture as well as used for transgenics.

The BP and LR reactions are made possible by the clonase enzymes that are provided with each kit. Cloning efficiency is high due to two selections incorporated in the system, the presence of antibiotic resistance and the presence of the ccdB gene in the unwanted clone. This gene causes lethality in standard lab strains of E. coli, and so the byproducts of the reactions, which contain the ccdB gene, are not recovered (Figure 8).

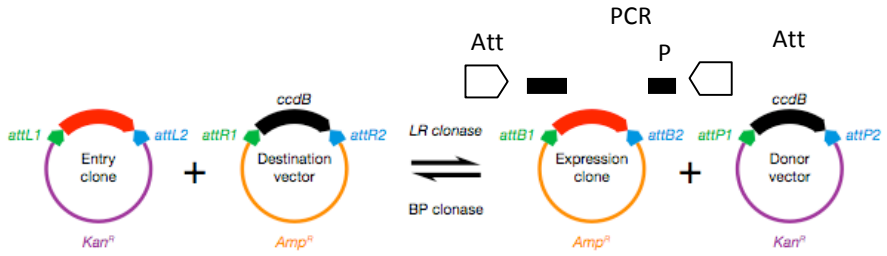

Figure 8. Gateway Recombination Cloning Technlogoy (Invitrogen).

The BP reactions were carried out according to Invitrogen's protocol. Stitched miniprepped constructs were used in the BP reactions with a pDONR vector: 7µL of DNA, 1µL of 150ng/ µL pDONR, and 2 µL of BP clonase mix were incubated at 25°C overnight. 5 µL of each BP reaction were subsequently transformed into Invitrogen's DH5α Max Efficiency Cells, and plated unto LB + Kanamycin plates (50µg/ml). Colonies were inoculated and miniprepped by Qiagen's Qiaprep Spin Miniprep Kit. Putative positive clones were analyzed by restriction digests.

The LR reactions were carried out according to Invitrogen's protocol. The previous pENTR clones obtained from the BP reaction were used LR reactions with both pUAST V5 6xHis and pUAST GFP as destination vector: 7µL of pENTR DNA, 1µL of 150ng/ µL pUAST-tag, and 2 µL of LR clonase mix were incubated at 25°C overnight. 5 µL of each LR reaction were again transformed into Invitrogen's DH5α Subcloning Efficiency cells, and plated unto LB + Ampicillin plates (50µg/ml). Colonies were inoculated and miniprepped by Qiagen's Qiaprep Spin Miniprep Kit. As previously, putative positive clones were analyzed by restriction digests.

Positive clones were maxiprepped with Qiagen's Plasmid Maxi Kit. The DNA obtained was sequenced at Yale's DNA Analysis Facility and verified by the lab by constructing contigs of the sequence reads using the program Sequencher.

Generation and Mapping of Transgenics

The maxipreppred DNA for construct pUAST-DER$^{SI\text{-}IVa}$-GFP was sent to Genetic Services, Inc. (Cambridge, MA) to be injected into w^{1118} embryos. The resulting larvae were returned to us for transgenic screening. Surviving flies (G_0) were single-pair mated to 3-4

w^{1118} males or females, depending on the sex of the putative transgenic. The transgene confers pigmentation to the eye, whereas the wildtype stock of w^{1118} flies contains no pigment. Any progeny from the previous mating containing eye color should then also contain the transgene. These transgenic flies (F_1) were collected, and males crossed with females of the genotypes [w*; Sp/CyO (chromosome II); +/+] and [w*; +/+; Ly/TM3,Sb (chromosome III)], for mapping purposes. Heterozygous progeny from these matings (pUAS-DER$^{SI\text{-}IVa}$-GFP/CyO or pUAS-DER$^{SI\text{-}IVa}$-GFP/TM3,Sb) were outcrossed back to w^{1118} to determine the segregation pattern of the transgene versus the marker. This mapping technique is outlined in Figures 9 and 10. An example is carried out in which the transgene inserted in the second chromosome. Figure 9 depicts the transgene segregating away from CyO, the 2nd chromosome balancer, whereas Figure 10 depicts the transgene segregating independently of TM3,Sb, the 3rd chromosome balancer. Segregation away from the balancer indicates which chromosome the gene has inserted into, and independent segregation indicates which chromosome the gene has *not* inserted into.

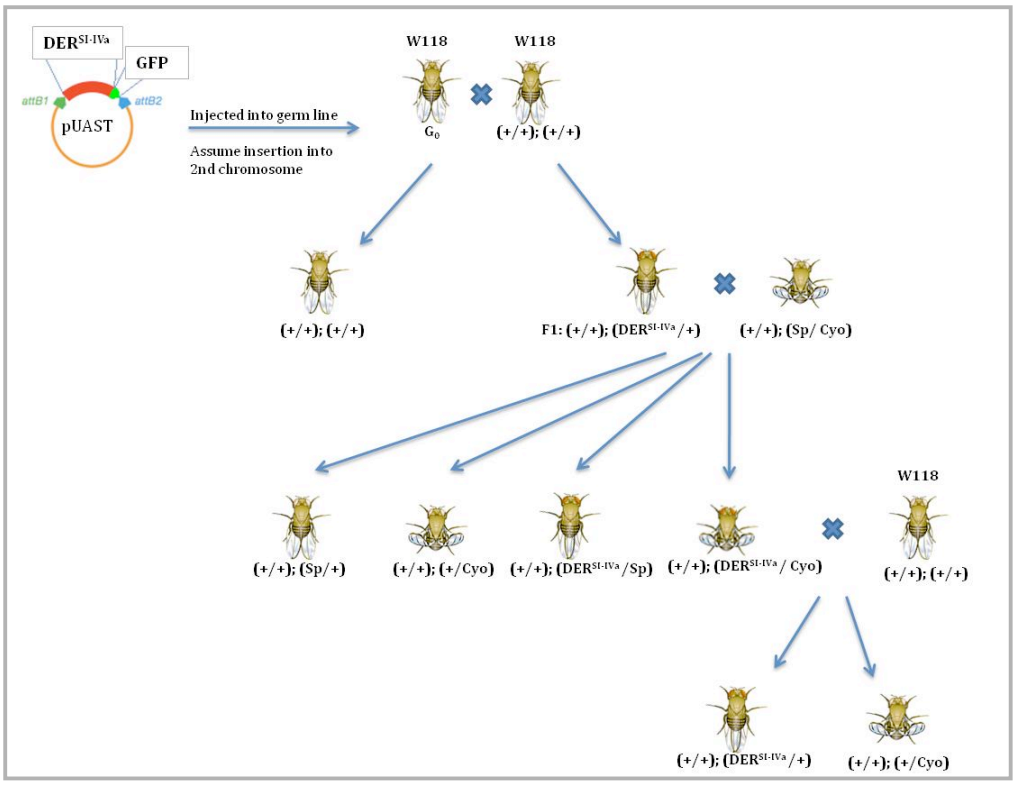

Figure 9. Transgene Mapping outline

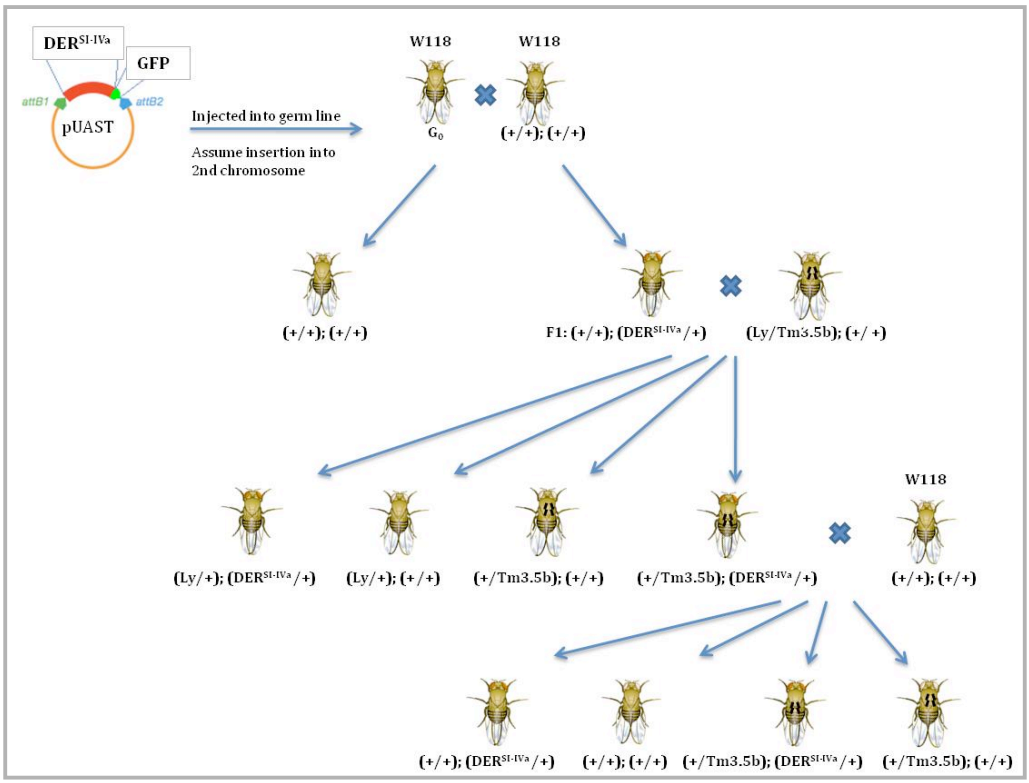

Figure 10. Transgene Mapping outline

Once mapped, stocks of the transgenic lines were created by first mating 4-5 transgenic males (pUAS-DER^{SI-IVa}-GFP/+) to females of the respective balancing stock (w*; Sp/CyO; +/+ or +/+; Ly/TM3,Sb). Female and male progeny of the genotype pUAS-DER^{SI-IVa}-GFP/ CyO or pUAS-DER^{SI-IVa}-GFP/TM3,Sb from this cross were mated to each other to create the final stock.

For insertions into the 1st chromosome (X chromosome), or the sex-determining chromosome, only F^{1} females contained the transgene. These females (pUAS-DER^{SI-IVa}-GFP/+), were mated by FM7 y w B males. Female progeny of the genotype pUAS-DER^{SI-IVa}-GFP/ FM7, y w B were once again mated to FM7 y w B males to create the final stock.

Gain-of-function Studies with the GAL4 System

The main tool used in the misexpression (gain-of-function) studies is the UAS/GAL4 system (Brand and Perrimon, 1993). In this system, the gene of interest can be expressed in a desired pattern using the mated combination of a driver (GAL4) and a responder (UAS) construct (Figure 11)(Duffy, 2002).

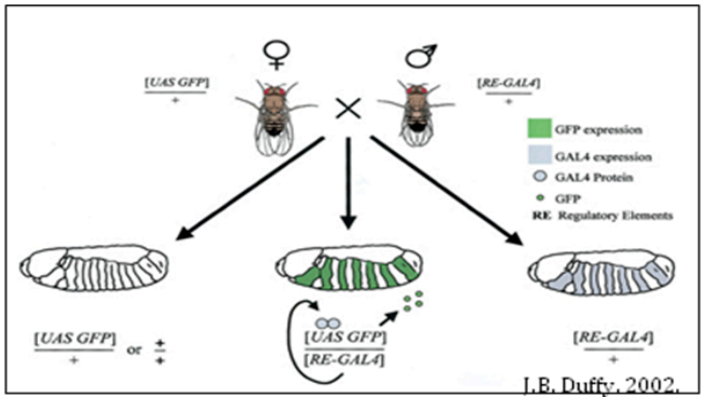

Figure 11. GAL4/UAS system

The GAL4 gene is linked to a regulatory element that expresses in a particular tissue, and so GAL4 is produced in this pattern. It will bind to the upstream activating sequence (UAS), which if present, should initiate the transcription of the linked gene (Brand and Perrimon, 1993). Expression can also be controlled temporally according to the temperature at which the fly crosses are carried out; the higher the temperature, the greater the levels of expression.

GAL4 CY2 and GMR drivers were used in this case, to induce misexpression of pUAS-DER^{SI-IVa}-GFP in the ovary and eye, respectively. 4-5 GAL4 driver females were mated to 5-6 pUAS-DER^{SI-Iva}-GFP males. For the line containing the construct insertion in the X chromosome, 3-4 responder containing females were mated to GAL4 driver males.

Adult Ovary Dissection and Mounting

Adult female ovaries were dissected in PBT, fixed in 3.7% formaldehyde with PBT, and mounted on a slide in 50% glycerol in PBS.

GFP Localization and Expression

Matured eggs from pUAS-DER^{SI-IVa}-GFP/Cy2 were examined for dorsalization phenotypes usinhg dark field microscopy on a Zeiss Imager.Z1 (20x). Stage 10 ovaries were then examined using a fluorescent Zeiss Imager.Z microscope with Apotome at 20x or 40x,

to observe the localization of GFP. Images were captured using a Zeiss Axiocam and were processed with Zeiss's Axiovision software.

Eye Dissection and GFP Localization

Adult male heads were cut off using a razor, and examined under a Zeiss Axiophot dissection microscope, both in bright field and fluorescence, to examine rough eye phenotype (rated from 1-3) and the presence of GFP. Images were taken with a Carl Zeiss AxioCam camera.

Co-transfections and Protein Detection

An S3 *Drosophila* cell line was maintained using Schenider's media with 12.5% FBS. Cells were seeded in a 6-well plate at 8 x 10^5 cells/well in 2mL of media, and transfected two days after seeding using a Qiagen Effectene Transfection Kit. Kek1-GFP, receptor-V5, and Arm-Gal4 (ubiquitous driver) were co-transfected into each seeded well. About 130ng of each DNA construct were co-transfected as depicted in Figure 12 below.

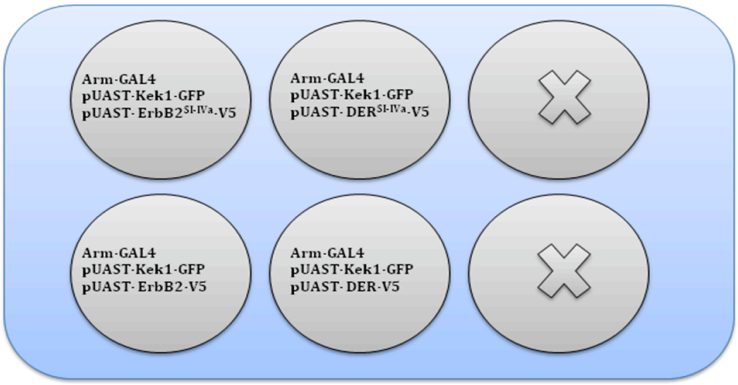

Figure 12. DNA combinations for co-transfections

50 microliters of each transfection were spun down using a tabletop centrifuge for 5 minutes at room temperature. Pellets were taken up in 40 microliters of 1x Loading buffer in 1x PBS. Samples were run in the same pattern on two 8% SDS-PAGE gels, according to the protocol adapted by C. Ernst for the Duffy Lab. Transfer was done with nitrocellulose membranes. One membrane was probed with monoclonal mouse anti-GFP antibody

(Clontech) diluted 1:1000 with TBST, while the other was probed with monoclonal mouse anti-V5 antibody (Invitrogen), diluted 1:2000 with 5% NFDM in TBST. Goat-anti-mouse secondary antibody was subsequently used for both gels, diluted 1:20,000 with 5% NFDM in TBST. The detection substrate used was a 1:1 peroxide:luminal solution, diluted 1:2 with water. Blots were developed using an X-omat and Kodak film.

Co-Immunoprecipitation (In Progress)

Using this technique, it will be possible to assay the interaction between the chimeric receptors and Kek1. This approach uses an antibody to immunoprecipitate the target protein (Kek1-GFP in this case), and co-immunoprecipitate any proteins that are interacting with it (i.e. DER-V5), as illustrated in Figure 13 below.

Drosophila S3 cells will be co-transfected in the previously outlined manner. On the third day from transfection, cells will be pelleted at 2000rpm for 2 minutes, then lysed using Fehon Buffer. After the insoluble fraction is removed, the supernatant will be incubated with the precipitating antibody, anti-GFP, overnight at 4°C. Total lysate samples of 50 µL will also be pelleted from each transfection, to be used as controls later.

Protein A Sepharose slurry will be added to each sample, followed by numerous washes. IP samples will be loaded unto an 8% SDS-PAGE gel along with the total lysate samples. Gel proteins will be transferred unto a nitrocellulose membrane, which will subsequently be probed with anti-V5, stripped, and re-probed with anti-GFP.

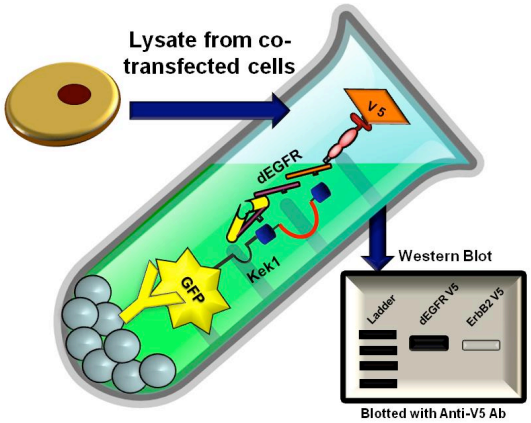

Figure 13. Co-IP schematic

RESULTS

A series of chimeric DER/ErbB2 constructs were designed and attempted. Two of these, IV (ErbB2^{SI-IVa}) and VI (DER^{SI-IVa}), were successfully generated and cloned into pUAST-V5 and pUAST-GFP expression vectors. To check for protein activity and localization, misexpression-based functional studies were done on transgenic flies expressing DER^{SI-IVa}-GFP. Based on these results, it was determined that the chimeric protein has activity and appears to localize properly. *Drosophila* S3 cells in culture were then co-transfected with ErbB2^{SI-IVa} and DER^{SI-IVa}, along with Kek1, in preparation for a co-immunoprecipitation assay. Cell lysate was successfully assayed by Western Blot and both Kek1 and the receptor variants were demonstrated to be expressed in S3 cells in culture.

Design of a Series of Chimeric DER/ErbB2 Constructs to Identify Sequence Elements that confer Kek1 Binding

A series of nine chimeric constructs were designed, of which a schematic representation can be seen in Figure 14 below. 5' and 3' primers were designed for each of the fragments composing a construct. Table 1 outlines the fragments of each construct, along with their respective sizes (basepairs), 5' and 3' primers, and the Tm's for the PCR reactions used to create them.

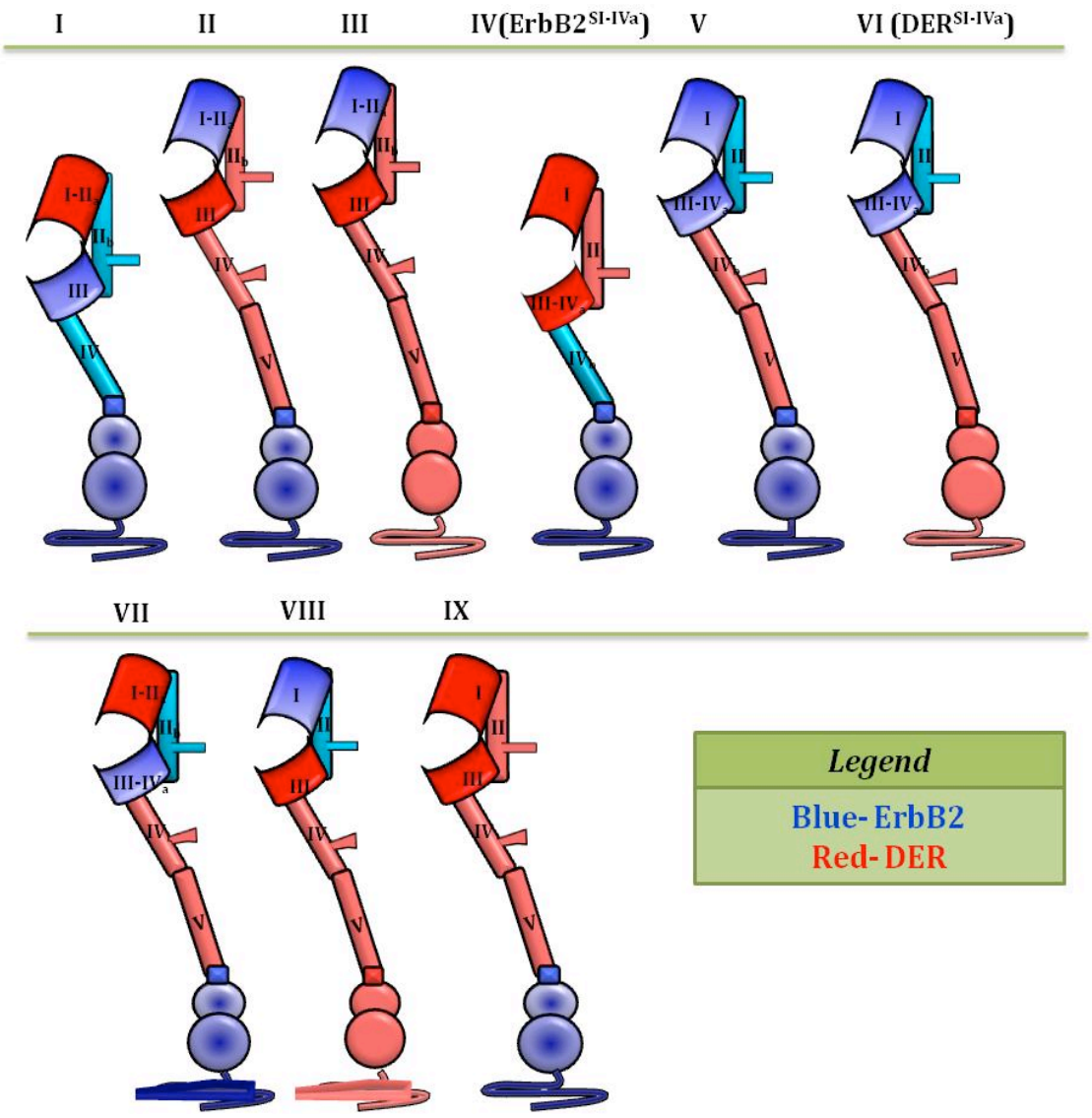

Figure 14. Schematic of DER/ErBb2 chimeric swaps (refer to text for details)

Table 1: Construct fragments, sizes, and primers

Construct	Fragments		5' Primer	3' Primer	Tm of rxn °C
I (4166)	IA (955)		W95	W79	60
	IB (3211)		W80	W92	60
II (4422)	IIAB (2486)	IIA (756)	W91	W87	60
		IIB (1730)	W88	W83	60
	IXB (1936)		W84	W92	62
III (4284)	IIIA (756)		W91	W87	60
	IIIB (3528)		W88	W96	60
IV (4166)	**IVA (1882)**		**W118**	**W126**	**60**
	IVB (2275)		**W125**	**W121**	**60**
V (4431)	VAB (2495)	VA (1692)	W91	W85	60
		VIIC (803)	W86	W83	62
	IXB (1936)		W84	W92	62
VI (4293)	**VIA (1692)**		**W91**	**W85**	**60**
	VIB (2601)		**W86**	**W96**	**60**
VII (4629)	VIIAB (1890)	VIIA (955)	W95	W79	60
		VIIB (935)	W80	W85	60
	VIICD (2739)	VIIC (803)	W86	W83	60
		IXB (1936)	W84	W92	62
VIII (4297)	VIIIA (1119)		W91	W89	60
	VIIIB (3178)		W90	W96	60
IX (4622)	IXA (2686)		W95	W83	62
	IXB (1936)		W84	W92	60

Note: Constructs IV (ErbB2$^{SI\text{-}IVa}$) and VI (DER$^{SI\text{-}IVa}$) were successfully generated and cloned into pUAST-GFP and pUAST-V5 6X His expression vectors as outlined in the Materials and Methods section. These constructs are highlighted in purple in Table 1. Construct IV was obtained using the Attb1.1 and Attb2.1 sites rather than Attb1 and Attb2 sites, which were used in designing primers for the rest of the series and generating construct VI.

Transgenic Fly Lines- Mapping

Construct DER^{SI-IVa}-GFP was sent to Genetic Services, Inc. for injection. G_0 larvae were sent to the lab, and the adults were single-pair mated to w^{1118} females or males. F1 progeny with eye color, a marker for the transgene, were selected and single pair mated with w^{1118} in order to map the transgene as described in Materials and Methods. Eight lines were obtained and mapped, with the results outlined in Table 2.

Table 2: DER^{SI-IVa}-GFP lines- Mapping Results

Line	Eye Color	Chromosome
1FV-MA	Medium Yellow	II
2FV-FA	Medium Yellow	II
13FNV-MA	Light Yellow	II
13FNV-MA	Dark Yellow	II
14FNV-MA	Light Yellow	III
16FNV-MA	Dorsal/ventral patches of expression	X (I)
26M-FA	Light Yellow/Medium Yellow	III
27M-MA	Light Yellow/ Medium Yellow	II

In Vivo Functional Studies

The activity of the chimeric construct was tested in each line by crossing transgene-containing flies with GMRGAL4 flies, as explained in Materials and Methods. GMR is an eye-enhancer, and thus misexpression effects are seen in this organ. DER overexpression in flies has previously yielded a rough eye phenotype, characterized by bubbling/blistering of the tissue (Brodu et al., 2004). The obtained rough eye phenotypes were rated from R0-R2, with R0 being no rough eye, R1 being weak rough eye, and R2 being strong rough eye. The

results of this study can be seen in Table 2 below. All the lines showed expression of GFP, indicating full translation of the protein.

Table 2: Results of room temperature GMR GAL4 missexpression

Line	Rough Eye	GFP
1FV-MA	R2	+
2FV-FA	R2	+
13FNV-MA	R2	+
13FNV-MA	R2	+
14FNV-MA	R0	+
16FNV-MA	R1	+
26M-FA	R1	+
27M-MA	R2	+

Images indicative of the overall results can be seen in Figure 15. DER-GFP containing flies were also tested as a control.

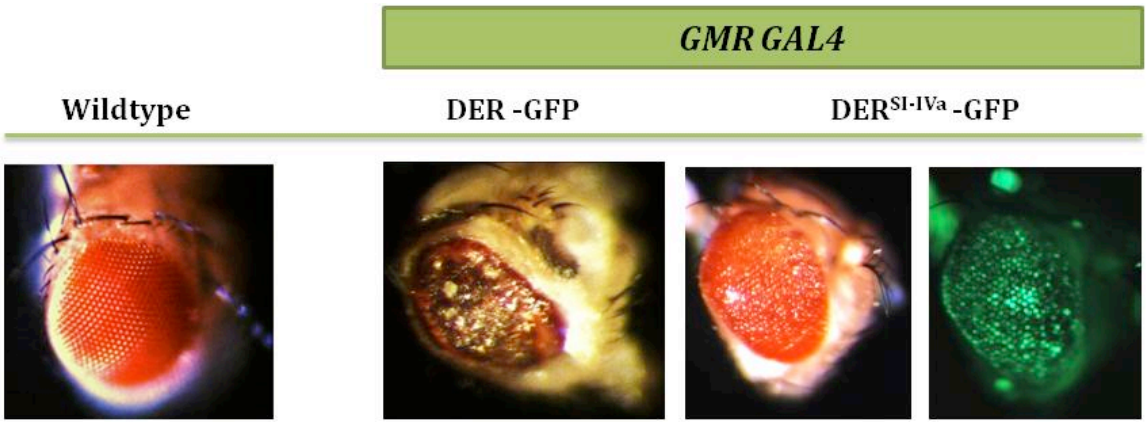

Figure 15. GMRGAL4 driven chimeric phenotypes in the adult eye

The activity and ability to localize of the chimeric construct were then tested in each line by crossing transgene-containing flies to Cy2 GAL4 flies, as explained in Materials and Methods. Cy2 drives expression in the follicle cells from stage 7 on (Duffy, 2002). Phenotypic effects are seen in both the chorion structure and in the protein localization during stage 10 of oogenesis. DER overexpression in flies has previously yielded a dorsalized chorion phenotype, characterized by fusion of the breathing appendages on the dorsal side of the egg (Figure 16)(Queenan et al., 1997). Additionally, overexpressed DER-GFP localizes to the apical membrane of the follicle cells surrounding the oocyte (Figure 17).

With crosses set up at room temperature, the misexpression of the chimeric construct was lethal in 6 out of the 8 lines tested. Dorsalization was seen in the 2 surviving lines, along with apical follicle cell localization (Table 3).

Table 3: Results of room temperature Cy2 GAL4 misexpression

Line	Chorion	Stage 10 follicular localization	GFP
1FV-MA		**LETHAL**	
2FV-FA		**LETHAL**	
13FNV-MA LY		**LETHAL**	
13FNV-MA DY		**LETHAL**	
14FNV-MA	Dorsalized	Yes	+
16FNV-MA	Dorsalized	Yes- Particular follicular cells	+
26M-FA		**LETHAL**	
27M-MA		**LETHAL**	

Images indicative of the overall results can be seen in Figures 16 and 17. DER-GFP containing flies were also tested controls. Dorsalized phenotypes ranged from normally dorsalized (similar phenotype to that of overexpressed DER) to severely dorsalized, in which the appendages are barely notable.

Line 16FNV-MA stage 10 ovaries had a particular follicular expression pattern, due likely to the location of chimeric construct insertion. A unique expression pattern was also seen in the dorsal/ventral patches of eye color expression in the mature transgenic flies.

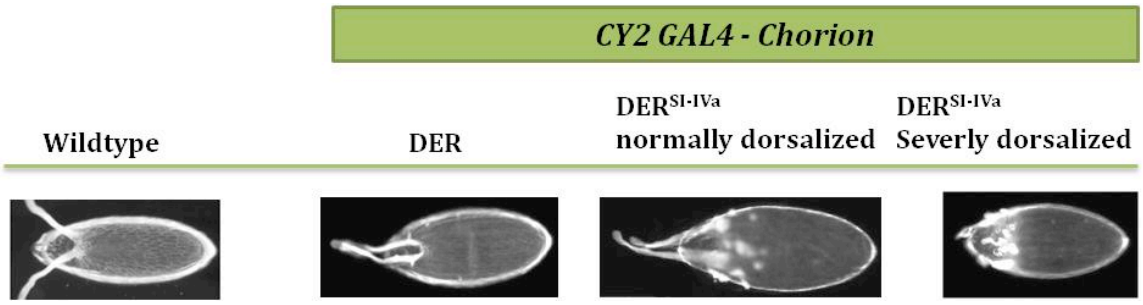

Figure 16. Chorion images of CY2GAL chimeric construct misexpression.

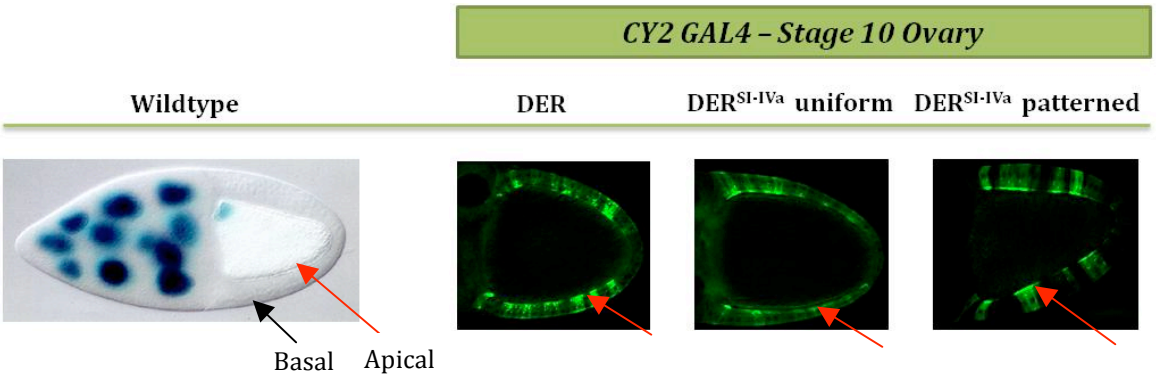

Figure 17. Stage 10 egg chamber images of CY2GAL chimeric construct misexpression.

Co-transfections –Blot Results

In preparation for the *in vitro* co-immunoprecipitation assay, co-transfections were carried out with DER-V5 + Kek1-GFP, ErbB2-V5 + Kek1-GFP, ErbB2$^{SI\text{-}IVa}$-V5 + Kek1-GFP, and DER$^{SI\text{-}IVa}$-V5 + Kek1-GFP. Cells were then pelleted, lysed, and examined using a Western Blot (Materials and Methods). Membranes were probed with anti-V5 antibody to visualize the receptors, and anti-GFP antibody to visualize Kek1 (Figure 18). Note that the Kek1 band appears to be missing from the DER/Kek1 co-transfection. However, this is likely due to an experimental error in the Western procedure rather than the transfection itself, as GFP was seen in those cells prior to pelleting.

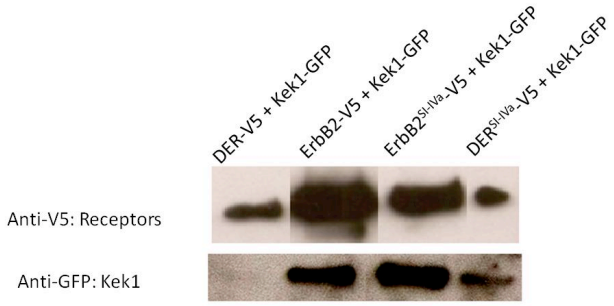

Figure 18. Anti-V5 / Anti-GFP Western blots of co-transfections

Co-Immunoprecipitation- Hypothetical Results

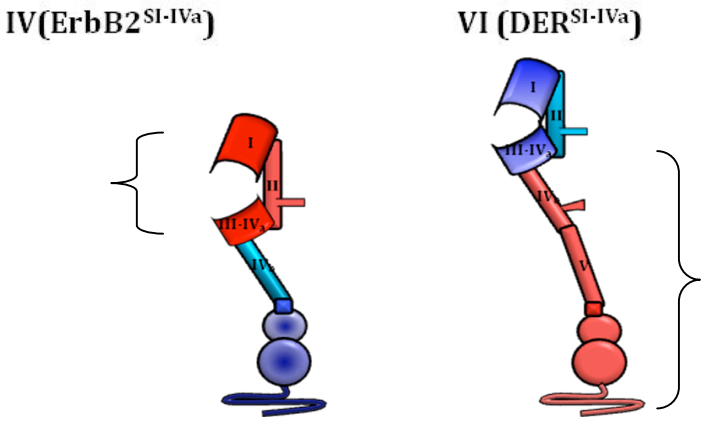

Figure 19. Structure of variants (Red represent DER, blue is ErbB2)

If extracellular domains IVb-V are necessary and sufficient for DER binding to Kek1, as they are believed to be, then $DER^{SI\text{-}IVa}$ will be able to bind Kek1 successfully in the co-immunoprecipitation assay (see Figure 19 for reference). Conversely, $ErbB2^{SI\text{-}Iva}$ will *not* be able to Kek1, as it is lacking these crucial DER domains. Using DER-V5 and ErbB2-V5 as positive and negative controls (see Materials and Methods for constructs used in co-transfections), the hypothesized Western blot results obtained from this assay can be seen in Figure 20 below.

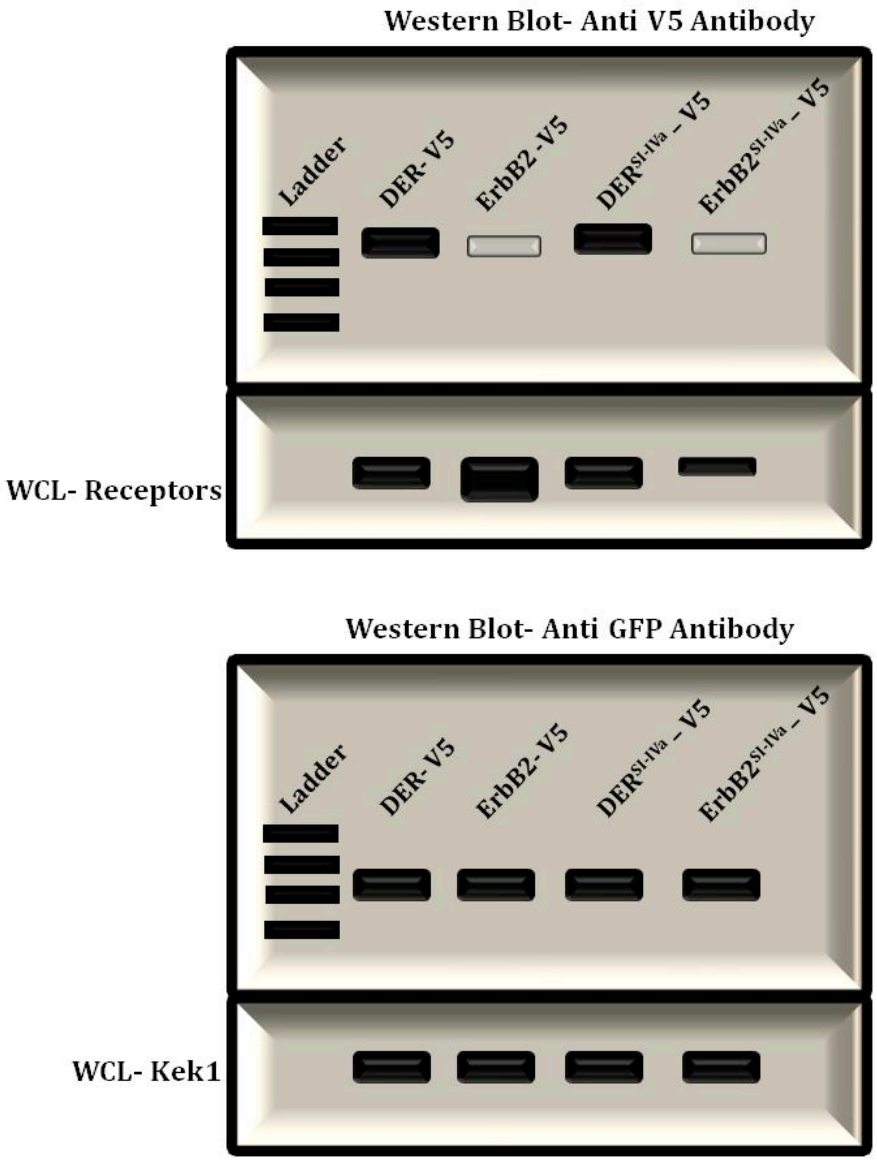

Figure 20: Hypothetical Western Blots for Co-Immunoprecipitation Studies

DISCUSSION

If properly understood, the DER/Kek1 interaction could potentially lead to the creation of a modified Kek1 or Kek1-based peptides specifically targeted for the human Epidermal Growth Factor receptors. As the members of the EGFR family in humans are often overexpressed in cancer cells, such a biologic could act to directly bind and inhibit receptor activation and signaling.

Previous studies done in the Duffy lab (K. Cook and J.B. Duffy, personal communication) have demonstrated that domain V of DER is necessary for the interaction between DER/Kek1. Its deletion from DER leads to a dramatic decrease in binding affinity for Kek1. Though informative, deletion studies like this one are not the optimal approach for exploring protein-protein interactions, as such deletions may also disrupt secondary and tertiary protein structure. Further experiments show that addition of domain V onto ErbB1 does not confer binding affinity. While swapping domains in this manner is a better way to examine protein-protein interactions, the swapping partners must be close in structure in order for proper protein folding to be maintained.

Recently published data from Alvarado et. al shows that ErbB1 and DER are not as structurally similar as was once thought. Rather, ErbB2 more closely resembles DER, both in the lack of an autoinhibitory domain II/IV tether, and in the presence of other stabilizing autoihibitory interactions between domains I/II and I/III.

Current functional studies are being conducted in the Duffy lab using domain swaps between Kek1 and other members of the LIG family in order to determine which domains of Kek1 are necessary or sufficient for binding to DER. I adopted a complementary approach to studying this interaction, by creating domain swaps between DER and its human homolog ErbB2 in order to establish which domains of DER are necessary and/or sufficient for binding to Kek1.

A series of nine DER/ErbB2 chimeric receptors were designed, two of which were successfully generated. Specific extracellular domain boundaries were obtained from Diego Alvarado. Upon his recommendation, domains II and IV were split into IIa/IIb and IVa/IVb, where the a notation is indicative of a portion of the domain that may in fact

belong to the previous domain based on structural studies. For example, it is believed that domain III actually encompasses the IVa fragment, and domain IV is made up of IVb only.

Stitching PCR was used to create the receptor variants, a method that often resulted in low yields of the desired products, thus it was difficult to obtain a large enough quantity of the stitched PCR product to be used in the Gateway BP cloning reaction. To overcome this, various permutations in temperature, primer/template concentrations, cycling conditions, and buffer reagents – Mg and DMSO were tried with varying degrees of success. To alleviate such problems in the future, increasing the Tm's of the primers might be an additional alteration. Increasing the stringency (annealing temp.) may help reduce the number of nonspecific bands, and thereby increase yield of the desired product. Other techniques that can be employed in the future are digestion ligations, and site-directed mutagenesis.

The UAS-DER$^{SI\text{-}IVa}$-GFP construct was the only one injected into flies for *in vivo* testing, as it was the only one successfully generated at the time. Eight independent fly lines were obtained and mapped to assure that expression patterns were due to the receptor's activity, not to the insertion location of the chimeric construct.

The UAS-DER$^{SI\text{-}IVa}$-GFP transgene was subsequently missexpressed in flies using the UAS-GAL4 system, specifically in the eyes with the GMRGAL4 driver and in the ovaries with the CY2GAL4. The rough eye phenotype that was obtained with GMR, and the dorsalized chorion phentoype that was obtained with CY2, both show that the chimeric protein is made properly and exhibits signaling activity. Similar phenotypes are also observed with DER overexpression in the same tissues. Upon examination of stage 10 egg chambers from the flies misexpressing DER$^{SI\text{-}IVa}$-GFP, it was found that the protein localizes to the apical side of the follicle cells, similar to that for DER-GFP. Collectively, these results suggest that this particular swap was successful in maintaining the general protein structure, activity, and localization. Further *in vivo* studies with the rest of the receptor variants in the series will continue to help define the sequence elements in DER critical for its interaction with Kek1.

It is worthy to note that there is some variation in the results (severity of dorsalization / pattern of localization). This is likely due to the chromosomal insertion location of the transgene in various lines. Expression of the transgene may be affected by local chromatin

structure thereby leading to phenotypic variation. Additionally, lethality upon misexpression with CY2GAL4 of so many insertions can be explained by the importance of proper regulation of DER activity throughout embryonic, larval and pupal development. In the future, in vivo studies with CY2GAL4 should also be conducted at 20°C, to reduce the level of misexpression and allow the survival of adult females for activity and localization studies in the egg chamber.

The co-immunoprecipitation studies have not yet been completed. However, it is hypothesized that upon examination of the four co-transfections, DER-V5(+ control) and $DER^{SI\text{-}IVa}$-V5 should both bind Kek1, whereas ErbB2-V5(- control) and $ErbB2^{SI\text{-}IVa}$-V5 should not. When blotted with Anti-V5 antibody, the IP should then yield bands for DER-V5 and $DER^{SI\text{-}IVa}$-V5, and no bands for ErbB2-V5 and $ErbB2^{SI\text{-}IVa}$-V5. If there is a band present for the $ErbB2^{SI\text{-}IVa}$-V5, then its intensity will correlate with the strength of that receptor's binding to Kek1. If binding is strong, it will mean that domains I-IVa of DER are sufficient to confer binding to Kek1.

Blotting with anti-GFP antibody of the IP should yield bands for every co-transfection, as Kek1-GFP will consistently be pulled down by the anti-GFP antibody in the protein A slurry, regardless of Kek1's interaction with the receptors. Whole cell lysate will also be examined on a Western blot, to ascertain that the individual proteins are being made and to estimate their amounts. If, for example, cells are not producing V5-tagged receptor this would invalidate any IP results, regardless of any putative interaction. Similarly, if a receptor is being made in great quantities but still no band is visible for the IP results, there is a greater likelihood that this variant lacks affinity for Kek1.

With the completion of the chimeric ErbB2/DER series and its testing both *in vivo* and *in vitro,* our understanding of the DER/Kek1 interaction will greatly increase. Using similar techniques it will then be possible to create an altered Kek1 molecule that can interact with the human receptors, while maintaining its direct inhibitory effect. This project has set the stage for the receptor's side of the DER/Kek1 story and has established a core set of techniques used to direct it. With the hope that ultimately this important and unique interaction may be used as a potential avenue for the development of Kek1 based cancer therapeutics.

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