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THE IMPORTANCE OF STUDIES USING THE DOMINANT NEGATIVE STRATEGY FOR FUNCTIONAL CHARACTERIZATION OF PROTEIN INTERACTIONS

By

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A THESIS

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DEDICATION

Dedicated to my parents, Michael David and Michaelle Leary Brown. Also, I would like to dedicate this to my siblings, Kourtney Ann and Keanon Anthony Brown.

ACKNOWLEDGEMENTS

I would like to thank my committee, Drs. Melissa Harrington, Theresa Szabo-Maas, Murali Temburni, and Hacene Boukari. I would also like to the Delaware State University Biology Graduate Program for giving me the opportunity to accomplish this work.

ABSTRACT

In the field of molecular biology, tools such as polymerase chain reaction, cloning, and the breeding of special knockout mice have been used to attain further understanding of proteins and molecular pathways. However, these techniques do not clearly explain the functions of proteins and their interactions. Dominant negative mutations are mutant proteins that are overexpressed and interact with the wildtype protein to inhibit its normal function. Applying this concept to research can be used as a tool for understanding the functions and roles of proteins and characterizing how these relate to protein interaction in diseases and normal biological functions.

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Chapter 1: Introduction

In 1953, Drs. James D. Watson and Francis Crick changed the field of biology forever with the discovery of the double helix structure of DNA. Their discovery was a milestone in understanding the importance of DNA and provided the foundation for investigating other questions, especially when discussing heredity. Biology as a whole was changed and the discipline of genetics would soon begin to flourish.

According to the National Human Genome Research Institute (NHGRI), although the Human Genome Project (HGP) had its practical and ideological origins in the 1980's, its true origins stretch back even further to 1911. The NHGRI argue that if not for famed geneticist Alfred Sturtevant and his gene map of *Drosophila melanogaster*, there would not have been a proper foundation for this project to come into fruition. As stated earlier, thanks to Watson and Crick in 1953, the molecular structure of DNA was discovered, however, the next crucial step in molecular biology was when Frederick Sanger developed techniques for DNA sequencing in the 1970's for which he won a Nobel Prize in 1980. It is also noted that he won his first Nobel Prize in 1958 for his research in protein structure.

The HGP began to take off in the 1980's, specifically 1986 when the United States Department of Energy began seeking to understand the mutagenic effects of radiation. Funding from Congress allowed this concept to go even further as in 1988 when the National Human Genome Research Institute was founded and collaborated with

Department of Energy under the direction of John Watson. In 1990, Francis Collins and David Galas published A New Five-Year Plan for the United States: Human Genome Project in *Science*. The goal of this project was to gain a further understanding of genetic inheritance. The advent of the HGP was the improvement of various techniques and technologies in molecular biology. These technologies include molecular cloning and polymerase chain reaction (PCR), which gives the ability to amplify the amount of DNA one is studying. The HGP also led to the discovery that the human genome possesses only 20,000-25,000 genes -- debunking the theory of 100,000-120,000 genes that was once believed (Pertea & Salzberg, 2010). However, the questions remain, what is there to be said about the proteins that these genes code for and their function? How do these proteins interact with each other? How would the discoveries of the HGP aid in the discovery of new treatments for that various medical ailments that plague humanity? What this project accomplished was a mapping of the thousands of genes in the human genome that would allow scientists to gain a greater understanding of proteomics.

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Chapter 2: From Genes to Proteins

Proteins belong to a class of molecules called macromolecules. Other molecules would be included in this category are carbohydrates, lipids, and nucleic acids. Each of these macromolecules is composed of smaller units or monomers that are held together via covalent bonds. In the case of proteins, they consist of chains of hundreds of amino acids held together by covalent bonds called peptide bonds. Proteins have a variety of functions that are predetermined by the nucleic sequences of the genes that code for them. These functions consist of transport, catalysis, signaling, aiding in DNA replication, and myriads of other unique functions.

Protein function is diverse, and understanding proteins involves understanding the basic synthesis of proteins. The master molecule, DNA, serves as a blueprint for an organism. The DNA is composed of chains of nucleotides that form nucleic sequences called genes that code for proteins. Genes are then transcribed to messenger RNA, which is also regulated by proteins, and then translated to a long polypeptide sequence in the ribosomes. Function comes into fruition when discussing the structure of a protein. When a protein is synthesized from the ribosome it is exists as a linear sequence of amino acids or primary structure. Then it forms its secondary structure that is composed of alpha helixes and beta sheets. In the tertiary structure, the protein folds into its functional three-dimensional structure. The structure and shape of a protein dictates the function (Carter, Rauvala, & Hakomori, 1981). Proteins are involved in almost every biological process.

Enzymes are proteins that are used to catalyze a reaction by lowering the activation energy requirements. Transport and channel proteins are used to transport nutrients, ions, or other macromolecules within an organism which are on the surfaces of cell membranes. Proteins can also be involved in signal transduction, which usually involves the use of trans-membrane proteins. One type of signal transduction occurs when a neurotransmitter acting as a primary messenger binds to a receptor protein, which then causes a response in the cell. The neurotransmitter receptor could open ion channels (ionotropic receptor) for quick responses or rely on secondary messengers (metabotropic receptor/G-protein coupled receptors) like kinases and phosphatases for a slower, modulatory effect (Carter, Rauvala, & Hakomori, 1981).

Transmembrane proteins are special because their processing involves translocation of the newly synthesized protein encoded across the ER membrane. An ER signal sequence included in the sequence is recognized and captured by the signal recognition particle or chaperone protein, which directs translocation of the protein across the ER membrane. Many transmembrane proteins, especially those that are destined to embed within the plasma membrane are extensively modified post-translationally. Post-translational protein modifications occur in the Golgi apparatus and causes structural changes that can affect the functionality of the modified proteins in important ways (Alberts et. al, 2008). The addition of different chemical groups to a protein can be a form of post-translational modification. One common and important modification is the glycosylation of these membrane proteins which occurs due to the action of enzymes called glycosyltransferases. There are two types of glycosylation, N-glycosylation and O-

glycosylation. N-glycosylation occurs when oligosaccharide chains are added to the amide nitrogen on asparagine side chains. However in O-glycosylation, oligosaccharide chains are added to the hydroxyl oxygen of serine or threonine residues. One function of glycoproteins in cell membranes is cell-cell recognition (Carter, Rauvala, & Hakomori, 1981).

Another example of post-translational modification is the attachment of lipoate through the process of lipoylation. Lipoic acid is a vital component in the pyruvate dehydrogenase complex during the citric acid cycle (Carter, Rauvala, & Hakomori, 1981). Other post-translational modifications include the addition of smaller chemical groups. In the case of acetylation, the addition of acetyl groups to lysine or arginine residues. Acetylation works as a switch to activate or inactivate genes (Carter, Rauvala, & Hakomori, 1981). Phosphorylation is another important modification in which phosphate groups are added to the serine, threonine, or tyrosine residues by protein kinases. Phosphorylation is key in discussing signaling pathways, which is an important concept when referring to G proteins. Post-translation modifications are an important determiner of the functions of proteins, especially for proteins whose destination is in the plasma membrane.

Proteins are among the most important versatile macromolecules in an organism. The functions range from basic structural support to advance functions such as catalyzing reactions and signaling. The modifications of these molecules enhance the functions of proteins in order that vital biological process can be executed. The central dogma of

genetics provides a clear illustration of how a seemingly simple yet complex DNA molecule provides the basic architecture for an organism.

The complex machinery that is involved in the process of transcription and translation yields the basic components for life. Although these processes might seem perfect, they are not without faults and errors. Mutations can occur spontaneously that may jeopardize the health or life of an organism. The errors in repair or chemically induced problems can arise from a silent replacement of a code for an amino acid or a complete deletion of an entire segment of DNA. Mutations can be problematic. However, a thing that might be dangerous for an organism can be good for science. In the following section of this paper, dominant negative mutations will be explained in how they can answer questions of how we understand the functions of proteins and how they interact.

Chapter 3: Dominant Negative Mutations

In order to ensure proper function in a given biological process, it is known that proteins must interact with each other. However, the study of protein interactions is a relatively new area, so much remains unknown. Only recently have tools, approaches, and reagents been available to allow functional characterization of protein interactions on a molecular level. What are some strategies in molecular biology that are being used to investigate protein interactions? The tremendous advances made in molecular biology, that give us techniques that allow tinkering with and manipulating DNA, also provided new approaches to study proteins.

One of these new approaches uses manipulations of DNA in order to study proteins. It is a technique to engineer cells to contain so-called "dominant negative mutations". Dominant negatives are mutations in which the gene product adversely affects the normal wild-type product within the same cell. In 1987 Ira Herskowitz and his laboratory group published a study in the journal Nature showing how cloned genes altered to encode mutant products can inhibit the function of the wild type gene product, this in turn causes a deficiency in protein function (Herskowitz, 1987). In essence, what they observed was that the overexpression of the mutant gene product had the ability to dimerize with the wild type product causing an inhibition of function. It is analogous to building structures that are composed of suitable and defective building materials. If the composition of faulty

building materials exceeds the amount of competent materials, this leads to an unstable structure.

A study in 1991 published in the journal Genetics, examined what may have been one of the first natural dominant negative studies published. Rebekah S. Rasooly of Albert Einstein School of Medicine was studying a lethal (1) TW-6^{cs} mutation of the *Drosophila melanogaster*. L (1) TW-6^{cs} is described as a cold-sensitive recessive lethal mutation that affects both meiotic and mitotic segregation (Rasooly, New, Zhang, Hawley, & Bruce, 1991). This mutation is a dominant antimorphic allele of the *nod* (no distributive disjunction) locus, which encodes for a protein similar to kinesin. When the scientists sequenced the *nod* locus, they observed a single base change in the putative ATP-binding region of the motor domain of *nod* (Rasooly et al., 1991). This change led to recessive loss-of-function mutations and disrupted the segregation of non-exchange chromosomes in female meiosis. Although the paper does not refer to this mutation as a dominant negative mutation, it perfectly illustrates the fundamentals behind the idea. This mutation in a dominant antimorphic allele disrupts proper function of the segregation of chromosomes. This allows observation of the importance of the protein function and how it interacts with other proteins.

In the evolving field of molecular biology, novel innovations in techniques are constantly being developed. With the various techniques for cloning genes available, engineered genes provide the necessary tools for investigating protein structure and function. The conventional approach to this problem involves inactivating a gene in order to observe the effect that the gene product has on the cell (Herskowitz, 1987). It is noted

at the time that this technique was being used, that this technique was primarily used in simpler eukaryotic organisms, such as yeast, by targeted insertion. However, during that time it was not possible achieve the same results with this technique in complex eukaryotic organism such in the cases of mammalian systems (Herskowitz, 1987).

Since the Herskowitz review was published, the technique of engineering and gene knockouts in mammalian models has been refined and has led to obvious progress in the biomedical sciences. Despite the contributions work with knockout models have brought to science, there are limitations to this method. According to National Human Genome Research Institute, using knockout mice as an example at least fifteen percent of gene knockouts are lethal (Copp, 1995). This early death would not allow for the gene function in relation to human heath to be observed in adult mice. Copp et al (1995) also stated that knocking out a gene of interest might fail to produce an observable change or may implicates a different function in mice than what is observed in humans when the same gene is inactivated. A potentially better approach involves an cloning in an altered gene that encodes for a mutant protein. This protein would then inhibit the function of the wild-type protein therein causing a defect in the function of that protein (Herskowitz, 1987). Herskowitz described the meaning behind the term "dominant negative" as the "dominant" pertaining to fact that the phenotype is manifested in the presence of the wild type gene. The "negative" describing how it is involved in the inhibition of the protein. Dominant negatives can also be described in this case as antimorphs due to their antagonistic activity, as opposed to other mutations, which simply decrease gene product activity thus being called "hypermorphs" (Muller, 1932) .

Proteins are the nuts and widgets of an organism's machinery. In order to ensure that this machine has optimum function, it is imperative that these proteins interact with each other. Now introducing a dominant negative mutant to this system will not only produce an inhibition of the protein, but can also disrupt the concerted effort of other proteins that interact with the mutated protein. Researchers have taken note of these observations and used them in their experiments in order to elucidate causes behind the diseases that cause much suffering.

Constructing dominant negative mutants involves the use of fundamental molecular biological techniques. Cloning is the main technique involved in producing these mutations. In order to clone the desired sequences, one has to develop primers for cloning. Primers are short sequences of synthetic oligonucleotides that have been designed to serve as the reverse compliment of the region of template on the DNA that is desired for cloning. In the terms of constructing a primer for a dominant negative sequence, a specific part of the sequence is deleted in order for this protein to be expressed as desired. Cloning involves the introduction of that construct to a plasmid inside a host organism. Commonly used in this case would be a bacterial host, with every replication, more of mutant constructs would be replicated. The most common method for transfection is the use of a virus via viral transfection (Carter, Rauvala, & Hakomori, 1981).

The next question to ask is how exactly do dominant negative proteins work? What are their mechanisms of actions? Muhlbach published a review in *Viruses* 2009 that gave some answers to these questions (Mühlbach, Mohr, Ruzsics, & Koszinowski, 2009). The focus of the work in this review was on the herpes virus. Their goal in this paper was to

explain methods to map and identify the functions of essential viral proteins. In order to accomplish this goal, they utilized the technique of dominant negative mutations to elucidate the functions of these proteins. As an example of how this might work, in terms of enzymes, a mutation of the catalytic domain can lead to the binding of a substrate but there would be no conversion to a product (Mühlbach et al., 2009). In terms of phosphorylation, for kinases in which phosphorylation reactions dependent on substrate binding and homo-dimerization, if one monomer carries a mutation within the substrate-binding site that could lead to the binding of a substrate being inhibited the reaction on the target molecule would be inhibited. Multicomplex proteins can be influenced by the mutations of different subunits within the protein complex. The binding of the dominant negative subunit competes with the wild type subunit in the complex, and incorporation of one mutated subunit could inhibit the ability of the complex to function as a whole (Mühlbach et al., 2009). Also, dominant mutations of transcription factors block the binding site for wild type transcription factors thus inhibiting downstream gene expression. In the Mühlbach review, they used plenty of examples in describing how dominant negatives worked. One of the papers that they cite is a series of experiments that uses the endocytosis protein dynamin-2 (Szaszák et al., 2002). In this research, the mechanism of dynamin-2 action was observed during endocytosis of the G protein-coupled AT1A angiotensin receptor. The tissues that they would express these proteins in were Chinese hamster ovary cells (CHO). The C-terminal proline-rich domain (PRD) of dynamin-2 is believed to interact with many SH3 domain contain proteins which are instrumental in endocytosis and recycling of synaptic vesicles. In order to test this theory, they made a

dynamin construct with the PRD domain deleted (dyn2-ΔPRD). The SH3 domains of the anphisphysin II and endophilin I, binding partners of dynamin, were expressed with a N-terminal green fluorescent protein (GFP) tag (Fig. 1) which allowed them to track the movement of those proteins. What they found was that the deletion of the PRD inhibited the interaction of the dynamin binding partner and this reduced the efficiency of endocytosis (Fig. 2).

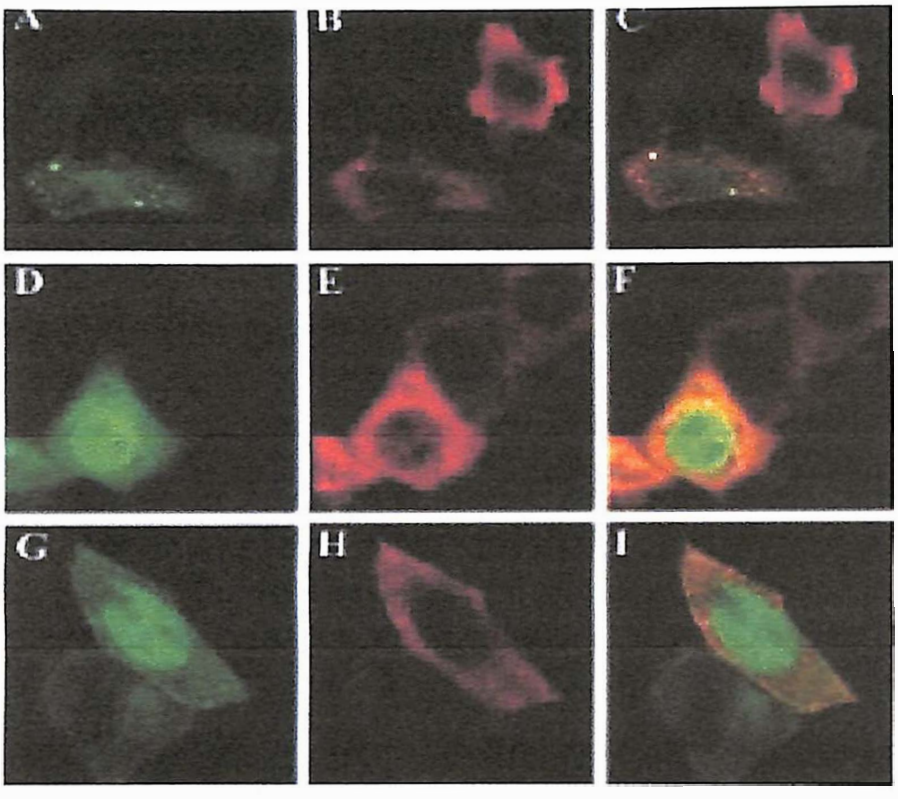

Figure 1: GFP tagged SH3-Endo, SH3-Amph, and SH3-Nck.
Subcellular localization of co-transfected CHO cells. (A-C) dyn2 combination with GFP-tagged SH3-Endo, GFP-tagged SH3-Amph (D-F), or GFP-tagged SH3-Nck (G-I) . SH3-Nck does not bind to dynamin as opposed to the Amph & Endo groups (Szaszák et al., 2002)

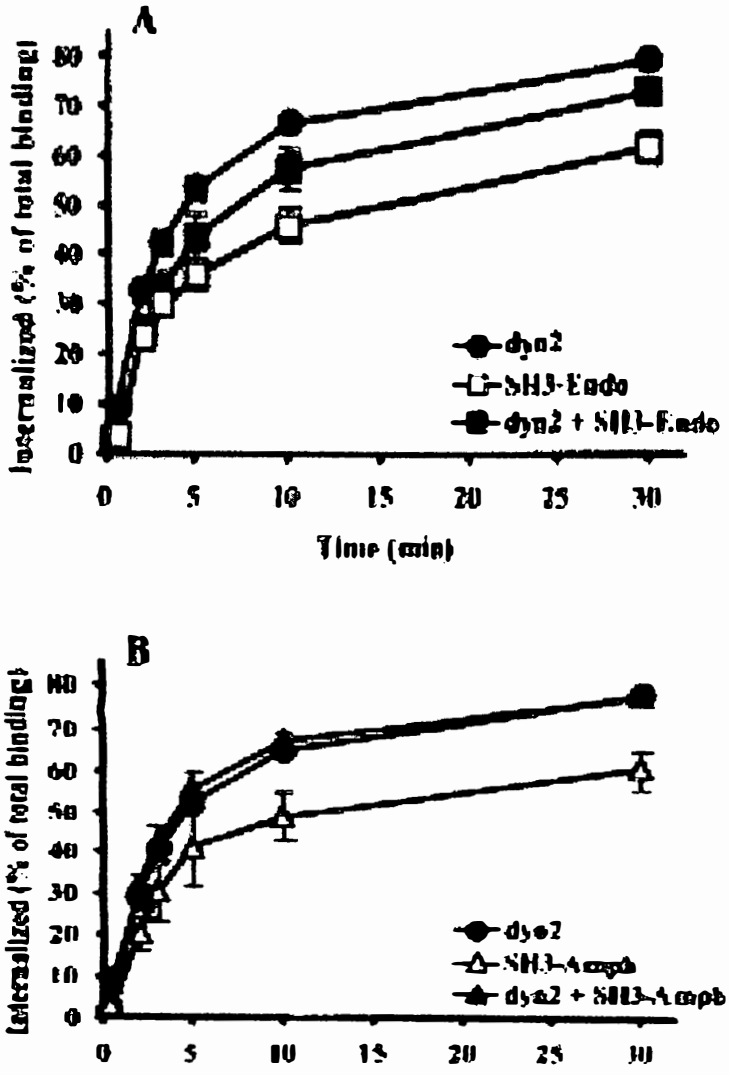

Figure 2: Overexpression of dyn2 interferes with the inhibitory effects of endophilin I and amphiphysin II SH3 domains on AT_{1A}-R endocytosis (A), 24 cell plates. Chinese hamster ovary (CHO) cells were transfected with the mutant cDNA of dyn2, the GFP-tagged SH3-Endo, and the GFP-tagged SH3-Endo and dyn2. (B), 24 cell plates. CHO cells transfected with dyn2, GFP-tagged SH3-Amph, and GFP-Tagged SH3-Amph/dyn2 (Szaszák et al., 2002).

The scientists concluded that the interaction between the dynamin-2 with SH3 domains of proteins amphiphysins and endophilins is essential for AT_{1A} receptor endocytosis. The significance of this mechanism is that it could show not only the importance in these proteins in dynamin-dependent endocytosis of other G-protein receptors in neuronal tissues but in non-neuronal tissues as well (Szaszák et al., 2002).

Other early studies with dominant negative mutations involved studies of tumor suppressor genes, such as a protein kinase R (PKR), in which dominant negative constructs expressed to study their roles in cancer cells (Li & Koromilas, 2001). The PKRΔE7 construct, in which exon 7 is deleted, was transfected in and expressed in HeLa S3 cells *via viral transfection. In the Li & Koromilas work, the* expression of the PKRΔE7 caused a dominant negative effect in which cell proliferation was halted. With this observation, the authors suggest that there may be a novel mechanism for controlling cell proliferation using spliced forms of PKR.

Another study uses dominant-negative to understand the pathology of diabetes mellitus 2. Type II diabetes is caused by the inactivation of IGF-1, a gene that codes for insulin receptors in skeletal muscle (Fernández et al., 2001). Transgenic mice were made expressing a dominant-negative construct of IGF-1, KR-IGF-IR in skeletal muscle. In order investigate this problem, high expression of the IGF-IR cDNA in skeletal muscle was achieved by the use of cloning via a plasmid vector with the muscle creatine kinase (MGK) promoter. These were just brief examples of past experiments using dominant strategies. Further into this paper, these concepts will be explained in sufficient detail.

In order to solve a problem, it is ideal to take a simple approach to arrive at a solution. As coroners perform autopsies to solve a cause of death, molecular biologists utilize similar concepts to understand protein function and their roles in interactions via pathways (Mühlbach et al., 2009). The purpose of this paper is to give a comprehensive understanding of this technique therein giving a greater appreciation for its use and how it contributed to advances in the biological sciences.

Chapter 4: Dominant Negative Strategies and the signal transduction of G-protein coupled receptors

Dominant negative strategies have been used in order to elucidate protein function and interaction. These experiments enabled further understanding of our knowledge of proteins. For example, G proteins are a class of proteins responsible for transducing signals from cell surface receptors across cell membranes to activate intracellular effectors (Ferguson, 2001). In a hallmark study, Gilchrist et al (1999) used dominant negative constructs, specifically mini-gene constructs of the C-terminal undecapeptides from G protein alpha subunits, to investigate the system of activation in muscarinic receptor activated potassium channels (Gilchrist, 1999). G-proteins work by the activation of G-protein coupled receptors (GCPRs), which leads to the interaction with their adjoining heterotrimeric G-protein. This G-protein construct consist of three sub-units: γ, β, and α. Upon activation of the receptor, this the heterotrimeric complex dissociates with the $G\beta\gamma$ subunits remaining bound to each other while the GDP is phosphorylated to GTP and bound to the α subunit. It is the GTP-bound α complex, which transduces the signal (Carter, Rauvala, & Hakomori, 1981).

Prior to this work, it was believed that magnesium played a major role in GTP binding to G proteins (Rudolph, Wittinghofer, & Vetter, 1999). This held true for other types of G proteins but not for all types. In the Gilchrist lab experiments, the biology of heterotrimetric G proteins was manipulated by developing a construct for the α subunit

variants by targeting the C-terminus of that protein subunit. In the terms of the role of Mg2+, it is a well-documented fact that GTP is bound as a complex with Mg2+ (Quilliam et al., 1994). In the dominant negative construct of $p21^{ras}$, a small G protein, it was shown that Mg had high affinity and irreversible binding in GDP thus giving clear role in its function (Kleuss, Raw, Lee, Sprang, & Gilman, 1994). However, when trying this approach with other G proteins of other families, scientists had noticed a lower affinity for Mg binding. For example, in the different variants of the non-heterotrimeric G proteins they were studying, G protein would bind to GDP-Mg with lower affinity than with GTP-Mg as seen in figure 3 and Table 1 below (Burstein & Macara, 1992).

Table 1. Constants for nucleotide and Mg^{2+} binding to $p25^{rab3A}$

The K_d is the equilibrium dissociation constant between $p25^{rab3A}$ and the indicated species (except for Mg^{2+}, which was equilibrated with $p25^{rab3A}$·GDP). k_{off} is the first-order dissociation rate constant; k_{on} is the second-order association rate constant derived from k_{off}/K_d. Values represent means of three separate experiments.

	GDP	GDP·Mg^{2+}	GTP	GTP·Mg^{2+}	Mg^{2+}
K_d (nM)	420	63	130	(46)*	4000
k_{off} (min^{-1})	0.63	0.011	0.22	(0.024)†	> 0.63
k_{on} ($\mu M^{-1} \cdot min^{-1}$)	1.5	0.18	1.7	0.52	> 0.16

* K_d for GTP[S]·Mg^{2+} (data taken from [27]).

† Value determined by subtracting $k_{cat.}$ obtained directly using method 2 (see the Materials and methods section) from $k_{off} + k_{cat.}$ obtained using method 3.

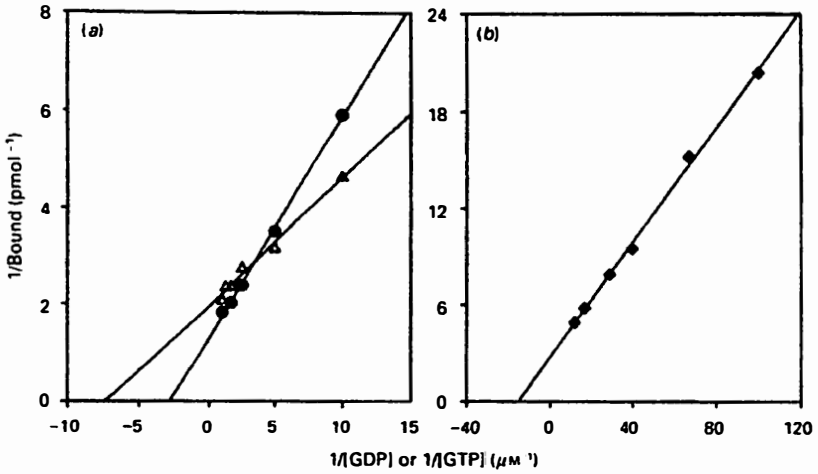

Figure 3: Equilibrium binding of GDP, GDP-Mg2+ and GTP to $p25^{rab3A}$: From the information given in Table 1, they used double-reciprocal plots to estimate the equilibrium binding. This was done to determine the dissociation constants for GDP (closed circle), GDP+Mg^{2+}(triangle), and GTP(closed circle) (Burstein & Macara, 1992).

Therefore this gave rise to the question of, besides Mg2+, what other mechanisms enable the proper function of heterotrimeric G proteins. For Gilchrist et al (1999), in order to study the function of G-proteins another method to induce dominant negative inhibition was used. Instead of targeting the Mg^{2+} binding sites of Gα, a minigene plasmid construct that would encode for a carboxyl-terminal peptide was developed. It was hypothesized that overexpression of this peptide would block cellular responses. "Minigene" plasmid vectors are constructs designed to express short polypeptide sequences (Gilchrist et. al, 1999). This peptide was tested using three isoforms of Gα: $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$. This hypothesis was tested by activating M_2 muscarinic receptors, which then activate the K^+ channels (GIRK) in human embryonic kidney 293 cells (HEK 293) cells via a G protein coupled pathway. In the presence of $G\alpha_i$, the carboxyl-terminal peptide did indeed result in inhibition of GIRK activity, as the presence of the peptide blocked the Ach-induced K+ current (Fig. 4).

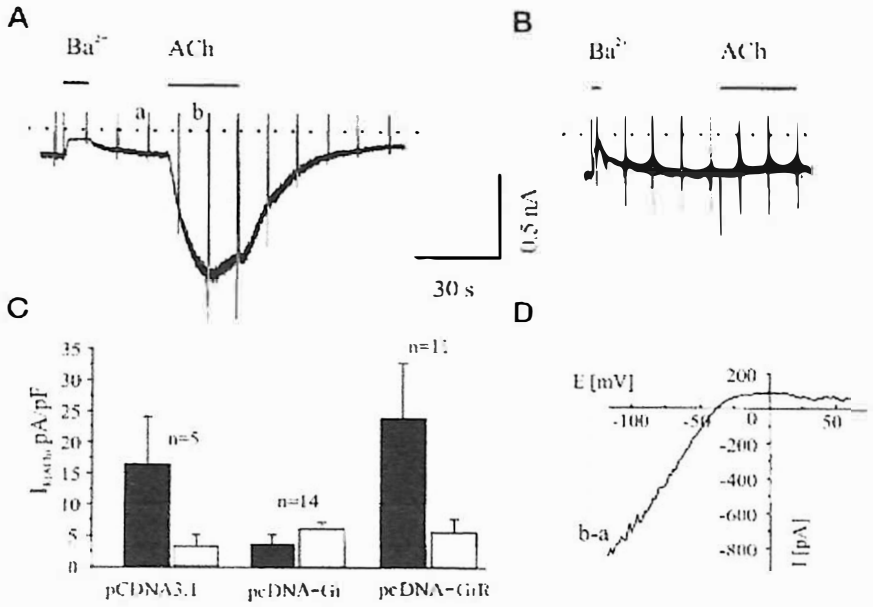

Figure 4 Minigenes encoding for carboxyl-terminal Gαi peptides inhibit mAChR activated I_{KACh} when expressed in HEK 293 cells: (A-B) Whole cell voltage clamp recordings measuring K^+ current in control (A) and with the Gαi peptide (B). (C), Gαi peptides inhibits mAChR when compared to the vector control or GiR (random order peptide). (D), voltage relation of the Ach-induced current between inward rectification and reverse potential near K^+ equilibrium typical for I_{KACh}. Acetylcholine evocked current (black bars) and barium-sensitive current (white bars)(Gilchrist, 1999).

As a control in the experiment, other minigene constructs were developed (Fig. 5). These constructs differed by possessing the Gα carboxyl peptide coded and arranged in a random order. This had no effect on the GIRK response. Also, the minigene construct was specific for the Gαi thus there were no inhibitory effects observed in the other two isoforms when stimulating the K^+ channel (Gilchrist et. al, 1999).

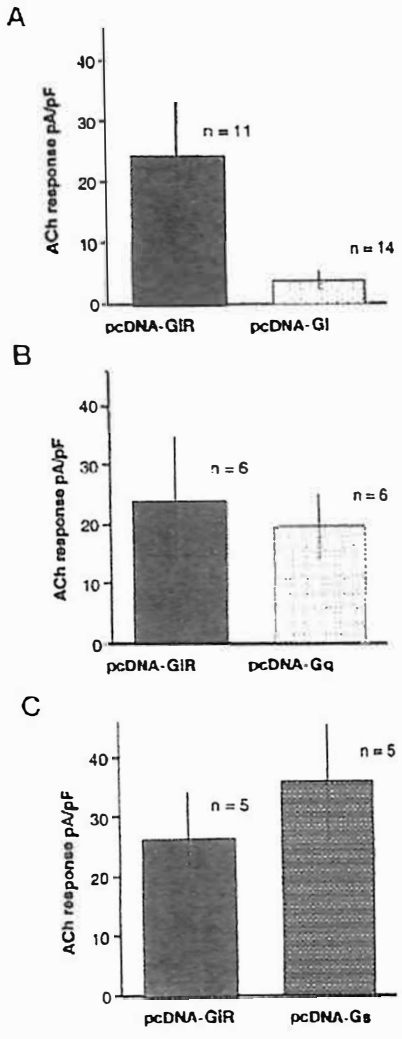

Figure 5: Transfection of $G\alpha_i$ carboxyl-terminal minigene inhibits M_2 mAChR activated I_{KACh}. $G\alpha_q$ and $G\alpha_s$ do not. (A), G_i inhibits mAChR-activated K+ current significantly when compared to the G_iR. (B-C), G_q and G_s do not inhibit mAChR K+ current (Gilchrist, 1999).

This experiment was paramount in understanding the function of how G-protein induced signaling works. It elucidated how the Gα is a critical factor in the mediation of the G-protein receptor and the selectivity of that receptor. Also another point that was made in this paper was the development of this minigene construct could be useful as a method for completely turning off G-protein mediated responses (Gilchrist et. al, 1999).

Since receptors themselves are proteins, the dominant negative strategy can be used their interactions with cellular effectors. In one study, dominant negative mutations in metabotropic glutamate receptors (mGluRs) are researched in detail. In a publication by Beqollari and Kammermeier in 2010, the mGluRs are described to form covalently linked homodimers and contain a large extracellular N-terminal ligand-binding domain called a Venus flytrap (VFT). This domain is responsible for the capture of glutamate at the synapse. In order for these dimers to form, disulfide bridges must be formed between the two monomers in order to dimerize the full-length receptors (Beqollari & Kammermeier, 2010). In their experiments, a dominant negative mutation of mGluR1 was expressed in order to study mGluR1 signaling.

In order to accomplish this experiment, they developed a mutant that expressed a truncated form of the mGluR1 receptor. The mutation was caused by the deletion of the cysteine rich region of the protein. The mGluR1 mutants co-dimerized with the full-length wild type proteins and were expressed on the surface of superior cervical ganglion (SCG) cells in rats. This was accomplished by injecting the cDNA for the mutant constructs into the rat SCG cells. They tracked expression of the gene using an immunofluorescence assay where the mutant protein with the first 590 deleted amino acids was incorporated with a

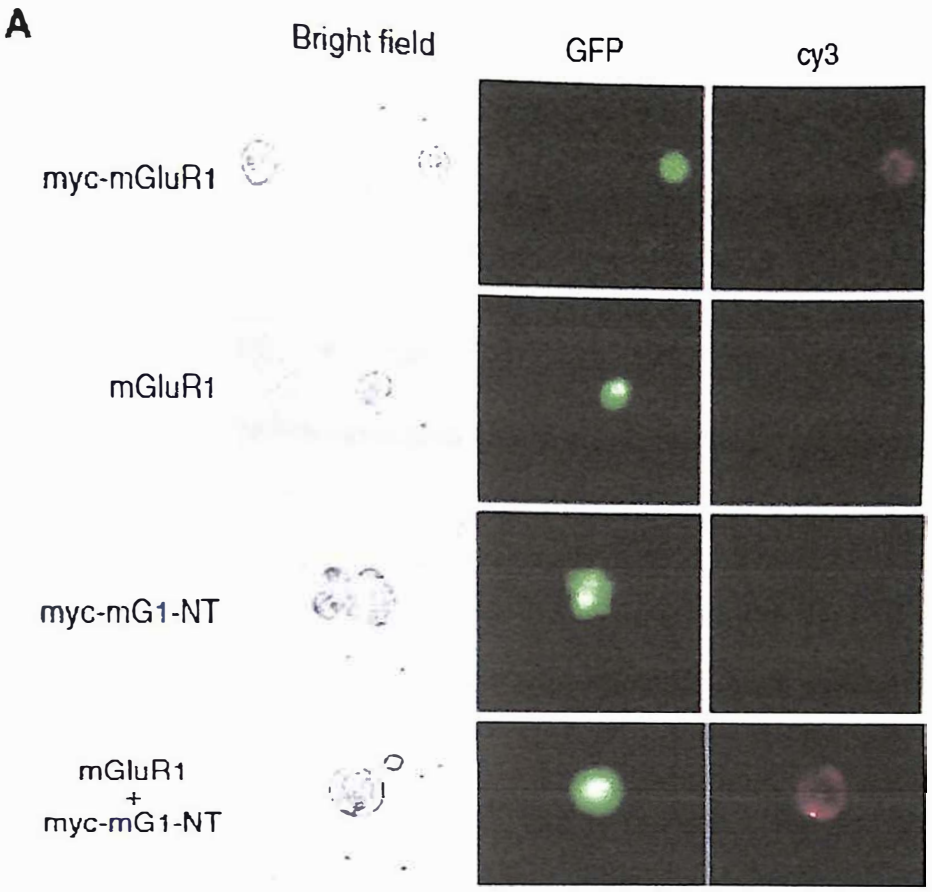

Figure 6: Full-length mGluR1 retains a myc-tagged, N-terminal mGluR1 construct on the plasma membrane. Cy3 labeling of the myc-tagged full-length mGluR1 can be observed on the surface of the membrane of SCG cells via antibody-labeling. Myc-tagged mG1-NT was not present on the surface when expressed alone. When myc-tagged mutant is expressed with the full-length mGluR1, cy3 labeling can be observed (Beqollari & Kammermeier, 2010).

myc-epitope tag. In order to determine if the construct was expressed on the plasma membrane, a Cy3-conjugated anti-myc antibody was used to detect the cell surface expression of myc (See Fig. 6).

The VFT mutants displayed dominant negative activity in respect to mGluR1 signaling. Since mGluR activation in SCG cells causes an inhibition of calcium current, they cold test the effect of the mutation by using the electrophysiological techniques to measure the amount of calcium current inhibition. In the one group using the mGluR1 wildtype alone, there was normal calcium current inhibition by glutamate. However when the mGluR1 wildtype was expressed with the mutant (mG1-NT), a decrease in calcium current inhibition was observed. This was measured by the glutamate-dose response (See Fig. 7).

They then wanted to test to see if mGluR1 could co-dimerize with another isoform, mGluR5. Therefore, spliced variants and mutant constructs (mG5-NT) of this receptor were also used in this experiment in order to note the extent that this VFT domain has on signaling. This was also done to give a better understanding of how the dominant negative mechanism of the VFT domain in mGluRs work in general. Once again, a glutamate dose response curve was used to measure the receptor behavior (Fig. 6). They displayed moderate dominant negative activity in comparison to the mGluR1 mutants/wildtype co-expressions. However, when the mGluR5 was co-expressed with mG1-NT, they observed strong dominant activity similar in mGluR1(Beqollari & Kammermeier, 2010).

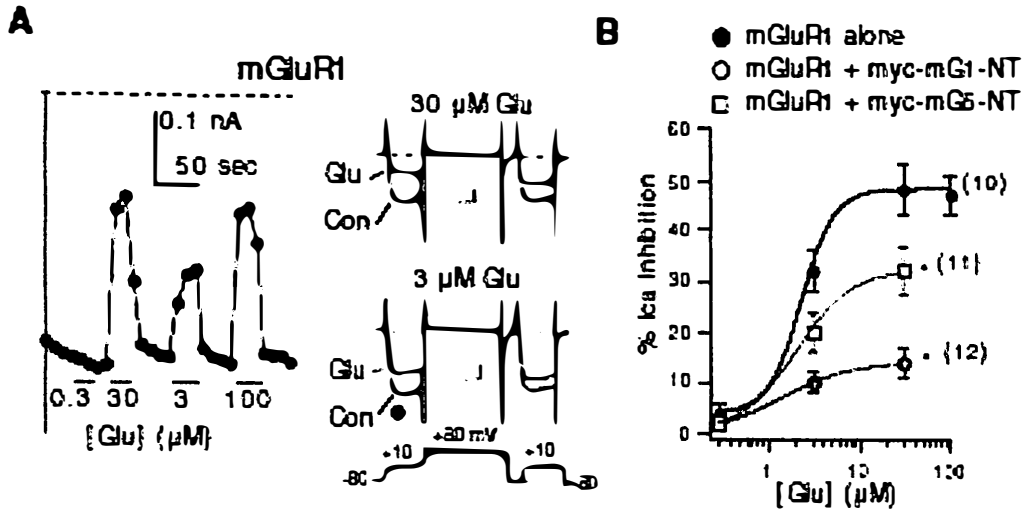

Figure 7: mGluR1-NT exhibits dominant negative effect on wildtype. (A) Voltage clamp experiment of wildtype mGluR1 showing reduction in peak Ca2+ current by different concentrations of glutamate (left) and sample current traces (right). (B) Pooled data for dose-response experiment showing the decrease in inhibition of mGluR1 activity observed in mG1-Nt mutant when overexpressed with wildtype and mG5-NT (Beqollari & Kammermeier. 2010)

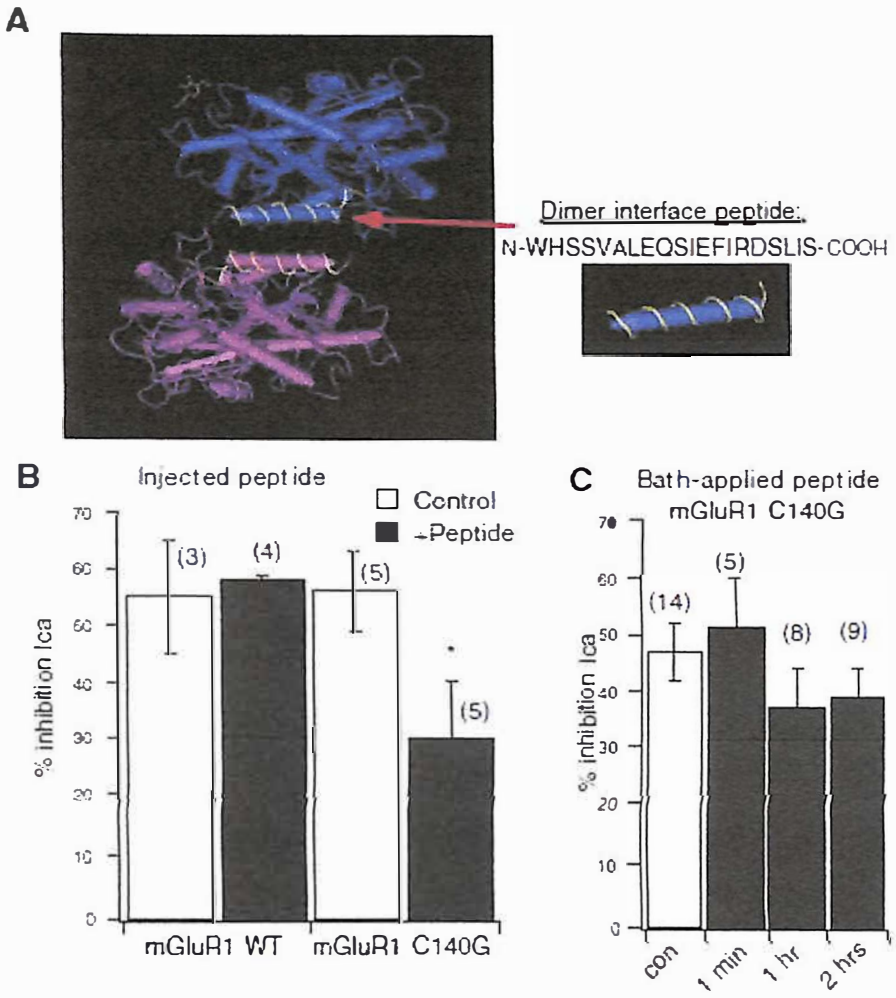

Figure 8: Injected and Bath-applied dimer interface peptide experiments. In the C140G mutants, the di-pep disrupted proper dimerization with the wildtype: **(A)**, schematic render of dimer interface peptide. **(B)** Group pre-injected with peptide showing significant difference from control in SCG cells. **(C)** Peptide applied during 2 hour bath of SCG cells (Beqollari & Kammermeier, 2010).

Utilizing dominant negative strategies in this experiment shed light on the mechanisms of mGluR1 signaling, knowledge that may be useful when applied to understanding many neurological diseases. The importance of the results with G proteins & receptors is that it sheds light on a fundamental signal transduction process that is crucial for functioning of all cells in all organisms.

Chapter 5: Dominant Negative Strategies and the Study of Disease

The purpose of this chapter is to give an overview of dominant negative strategies and their application to the research in disease mechanisms. In the areas of research concerning cancer, diabetes, and prions, it is commonly observed that the pathology of these diseases is often associated with problems with protein interactions. The dominant negative strategy employs clever methods to understanding the pathways in which proteins interact in these diseases underlying the probable cause for their pathology.

5.1: Diabetes

In the field of diabetes research, dominant negative mutations are used to investigate pathology and find new approaches in treatments. Diabetes mellitus, as it is known, is organized into two categories. Diabetes mellitus type 1 is an autoimmune disorder, which destroys □ cells within the pancreas so that they do not produce sufficient insulin to reduce blood glucose levels down to normal. This type is referred to "insulin deficient". In type 2, insulin is being produced, however, the insulin receptors do not respond properly to insulin binding, so that glucose is not taken up into cells. This causes the pancreas to produce more insulin in order to maintain proper blood glucose levels. Eventually the demands that the pancreas has been given cannot be met therein blood glucose levels continue to increase. A study by Goodyear and colleagues in 1995, suggests that the impairment of insulin-stimulated glucose uptake in skeletal muscle is accompanied by deficiency in insulin receptor signaling which likely contributes to decreased insulin

action. The decrease in signaling is caused by a decrease in insulin receptor phosphorylation (Goodyear et al., 1995). This is important because insulin stimulates the autophosphorylation of insulin receptors on tyrosine residues (Tavaré, O'Brien, Siddle, & Denton, 1988). In the study by Tavaré in 1988, using two intact cell lines as a model, it was observed that insulin causes a rapid increase in phosphorylation of tyrosine residues 1146, 10, and 1151.

This leads to many questions of on the nature of insulin receptors when studying type 2 diabetes. Insulin receptors are trans-membrane oligomeric glycoproteins that bind the insulin on the surface of target cells. In a paper published by Pi-Yun Chang in 1994, a dominant negative human mutant insulin receptor was expressed in the muscle of transgenic mice resulting in receptor kinase deficiency. Receptor kinase deficiency is a result of naturally occurring mutations in the receptor gene. These mutations manifest themselves in the form of missense mutations that affect the insulin receptor tyrosine kinase domains. In their experiments they generated two lines of transgenic mice that expressed the receptors in striated and skeletal muscle. The cDNA coding for the mutant receptors was microinjected into zygotes and then transferred into the oviducts of female mice. In order to accomplish their work, they first had to detect to see if the receptors were expressed on the muscle cells using immunodetection techniques. They assayed the function of the insulin receptors be using fasted, anesthetized mice and performing intraperitoneal injections of insulin. Following the insulin injections, they perform biopsies on the muscle type being tested. In the results it was concluded that overexpression of kinase-deficient human insulin receptor effects level of kinase activation (see Figure 9 next page);

impairment of the insulin-stimulated muscle receptor tyrosine kinase activity caused decreased insulin sensitivity; and through this the develop of these mutants and the success of the experiments, novel animal models can be used to study insulin resistance.

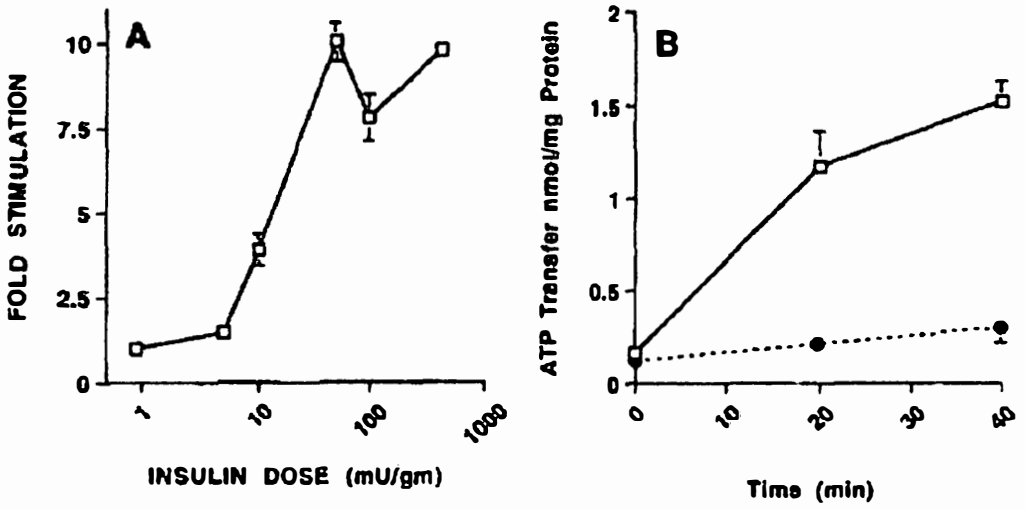

Figure 9: Skeletal muscle insulin muscle receptor tyrosine kinase activation after in vivo insulin stimulation. Non-transgenic mice (square), transgenic (circle). (A), dose-response curve using increased insulin doses in non-tg mice. Receptor kinase activity 30 minutes after insulin administration relative to kinase activity in muscle from mice who received no injection. Line for transgenic mice is at the baseline. **(B)**, kinase activity over time is diminished in transgenic mice compared to non-transgenic mice (Chang, et. al, 1994).

This technique has continued to be used to investigate type 2 diabetes in further detail. A paper by Ana M. Fernandez published in 2013, utilized similar techniques using transgenic mice in order to study type 2 diabetes. In this work, the insulin-like growth factor-1 (IGF-1) gene was co-expressed with a dominant-negative mutant insulin-like growth factor-I receptor (KR-IGF-IR) to study how type 2 diabetes is developed in humans. IGF-1 is a hormone whose effects are mediated by IGF-IR. It is instrumental in childhood growth and has similar functions as insulin in muscle. In IGF-IRs, like insulin receptors, it is a tyrosine kinase that signal when phosphorylated. If IGF-1 binds to an insulin receptor, then hypothetically glucose reuptake would be observed in the cells (Fig. 9). This hormone targets almost every type of cell in the body such as bone, liver, kidney, and others.

However in this experiment, KR-IGF-IR was overexpressed and the effect observed in striated muscle. The objective of this research was to observe whether or not the IGF-IR has any role in insulin reuptake (Fernández et al., 2001). The transgenic mice overexpressed the mutant KR-IGF-IR and it interacted with the endogenous wild type IR thus forming a hybrid receptor. They used the transgene MKR to generate seven independent lines of transgenic mice. They then used southern blot and RNase protection assays to reveal strong expression of human mutant IGF-IR in skeletal muscle. The dominant negative IR impaired both the IGF-1 reuptake and the insulin signaling in the striated muscle. The impact of the defective IR mutants was to increase blood glucose levels therein disrupting homeostasis, which they assessed by using a glucometer by

Although the purpose of this study was to gain a broader understanding of mGluR1 function and its role in signaling, this work also gives clarity to its role in protein interaction. The introduction of this paper discusses G-Protein association and activation, the importance of which is that mGluR1 are G-protein coupled receptors (GPCRS) so binding of a ligand to this protein activates a secondary response via G-proteins. These GPCRs are known to contain hepthatical domains that anchor them to them to the surface of the plasma membrane and mediate the association and activation of G-Proteins.

These hepthatical domains are linked to short extracellular cysteine-rich domains that are believed to be involved in the conformational changes necessary for the VFT domain to translate the ligand binding into G-Protein activation. In mGluR1 receptors, G protein activation will not occur unless bound by the ligand in both dimer subunits (Kammermeier & Yun, 2005). Therefore by truncating the region of the mGluR1which dimerizes with the full-length receptor the interaction with the G-protein is disrupted disabling activation thus inhibiting signaling. In this paper, the authors suggested that in order for there to be proper function and interaction, not only does their have to be full-length receptors but also there is a role in dimerization in the interaction. The investigated this by performing an experiment using a dimer interface peptide (di-Pep) to selectively disrupt mGluR1 signaling (See Fig. 8) . When they applied the peptide, either by injecting it into SCG cells or by application in the bath, the dimerization of the full length proteins were inhibited thus disabling proper function and reducing glutamate-mediated inhibition of the calcium currents (Beqollari & Kammermeier, 2010).

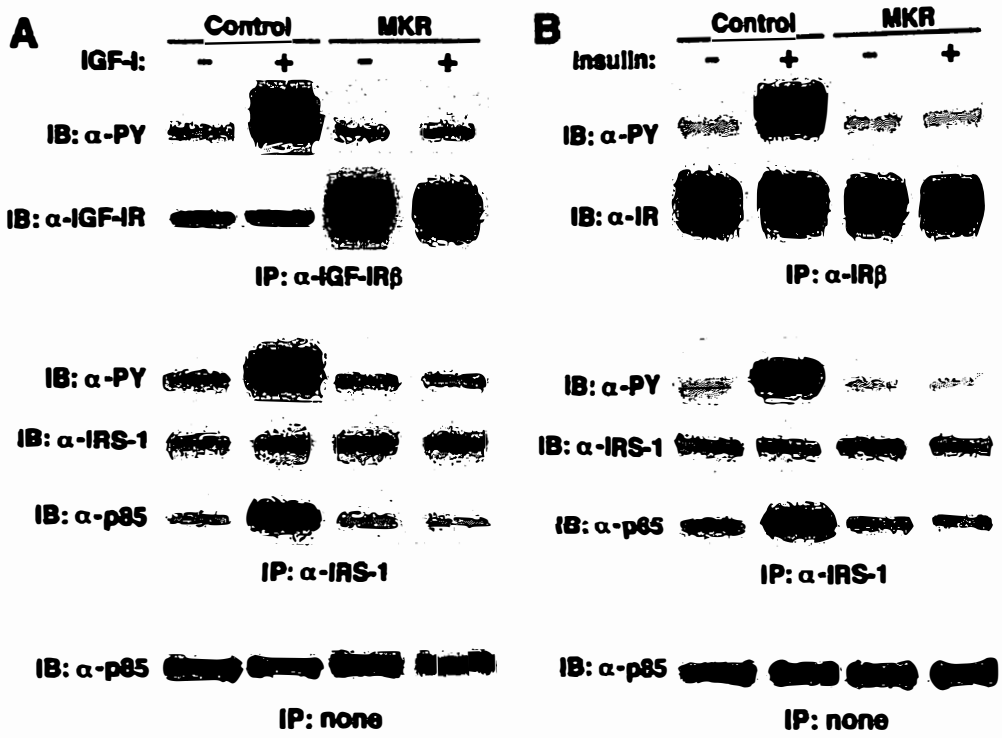

Figure 10: Overexpression of a dominant-negative IGF-IR impairs both IGF-I and insulin signaling in skeletal muscle. IGF-I and insulin stimulated signaling is inhibited in MKR mice. **(A),** IGF-stimulation, (upper panels), the skeletal muscle was subjected to immunoprecipitation of the IGF-IR, IRS-I (middle panels), Western blotting with anti-phosphotyrosine antibody, IRS-I antibody, and p85 antibody (bottom panel). **(B),** experiments were performed as described in A only with insulin (Fernández et al., 2001).

collecting blood from the mouse-tail vein. This was a accomplished after a time course IP injection of glucose to access the function of these receptors(Fernández et al., 2001).

In their findings, they found that overexpression of the dominant-negative IGF-IR impaired both IGF-I and insulin signaling. Since insulin receptors (IRs) and IGF-I receptors share the same signaling pathways, they determined that dominant negative IGF-IR affected the function of the IRs by evaluating the signal pathway in MKR mice versus the wild-type mice. In this experiment they observed that insulin induced phosphorylation was reduced in IRs and insulin receptor substrate-I, which is a protein involved in the transmission of both IRs and IGF-I (see Figure 10).

They also investigated how the impaired receptors affect the reuptake of glucose and impairs the glucose homeostasis of the MKR mice. In this part of the experiment, they explored the consequence of reduced IGF-I signaling and insulin signaling when measuring *in vitro* glucose uptake in extensor digitorum longus (EDL) muscle. They used a non-metabolizable form of glucose, 2-deoxyglucose, and showed that the MKR-impaired receptors reduced the amount of 2-deoxy-glucose uptake in response to insulin in muscle cells and also increased blood glucose concentrations (Figure 11).

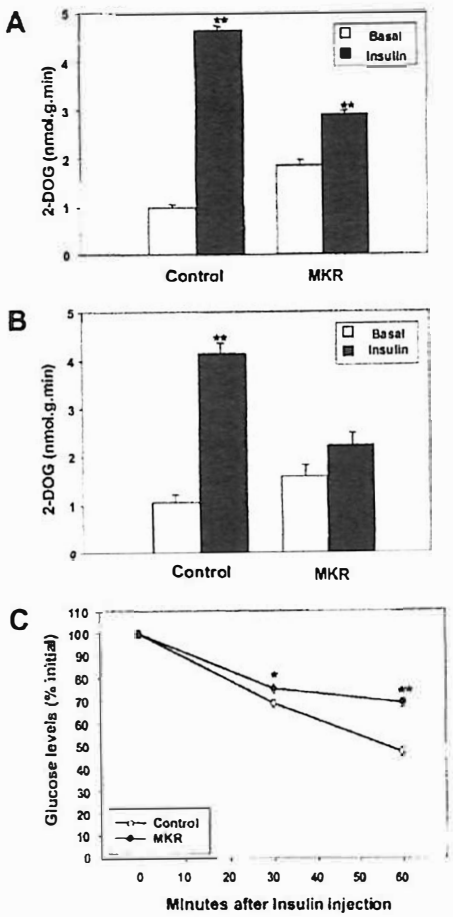

Figure 11: Impairment of glucose uptake in skeletal muscle leading to insulin resistance. (A), Impairment of insulin signaling on KR-IGF-1R. (B), Impairment of IGF-1 signaling on KR-IGF-1R. Reduction in 2-deoxyglucose (2-DOG) uptake in response to insulin. (C), Serum glucose levels higher in MKR 60 minutes following insulin injection (Fernández et al., 2001).

In the wild-type mice, an increased reuptake in blood glucose was observed after insulin. However in the MKR mice there was not much of a difference in glucose uptake or blood glucose levels in response to insulin. In order to check if this was not the result of a down-regulation of GLUT4, the main insulin-stimulated glucose transporter in cells, they checked immunoreactivity levels in whole muscle extracts and observed normal levels of the transporter. In terms of homeostasis, they determined that MKR mice displayed hyperinsulinemia at the early age of 2 weeks old. Hyperinsulinemia is the excess levels of insulin in the blood relative to glucose and is usually an early sign of type II diabetes. In later experiments this lead to noticing dysfunction in beta cell insulin secretion in the pancreas, in which 3 to 4 week old MKR mice had elevated levels of insulin (Fernández et al., 2001) (see Figure 12).

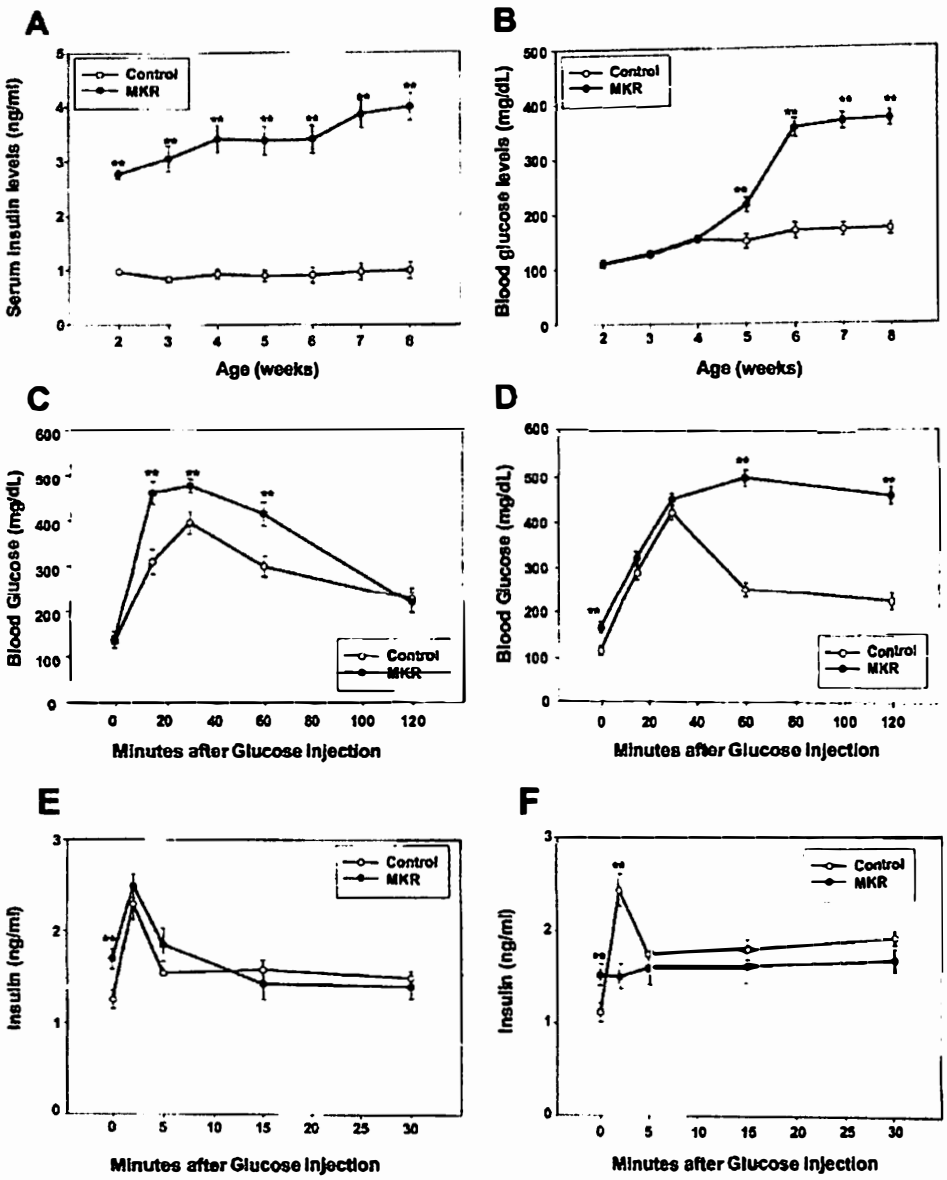

Figure 12: Progressive metabolic and beta cell disturbances in MKR mice: Glucose Injections. **(A)** Insulin and **(B)** glucose levels from 2-8wk old mice. **(C)** 3-4 week old mice or **(D)** 8-12wk old mice. Beta cell function. **(E)** 3-4 wk old and **(F)** 7-12 wk mice, insulin blood levels were measured (Fernández et al., 2001).

The main point in these series of experiments was to produce an excellent animal model for understanding the molecular mechanism behind the development of human type II diabetes mellitus. These experiments highlighted the primary differences between type II diabetes and pre-diabetes, with the former having the main characteristic of insulin resistance. Pre-diabetes is classified as a state of limbo between normal fasting blood glucose levels and higher than normal levels but not high enough to be classified as diabetes. Therefore, with the fundamentals of understanding this disease are still being developed, this can also offer better method of treating this problem that affects millions of people.

5.3: Channelopathy

Another current example of the uses of dominant negative strategies is in the study of Brugada syndrome. Brugada syndrome is an autosomal dominant cardiac channelopathy. Channelopathy can be defined as disturbed function of ion channel subunits of the proteins that regulate them. According to the literature, the mutations in the sodium channel $Na_v1.5$, which cause the disease result in misfolded proteins that do not allow proper trafficking of the Na channels to the membrane (Baroudi et al., 2001). In a paper published by Mercier in 2012, a trafficking defective mutant (R1432G*), which is caused by the misfolding of the pore-forming* □ □*subunit,* was co-expressed with the wild type $Na_v1.5$ HEK 293T cells. When the □1 subunit of the mutant channel was co-expressed with the wild type channel, a significant decrease in membrane Na current density was observed (Figure 13). Also, the expression of the mutant channels induced a

significant reduction in the membrane expression of wild type channels. However, when the □1subunit of the R1432G channel was not expressed, the mutant had no dominant negative effect (Figure 14).

When analyzing the data, it was observed that the absence of the β_1-subunit in the Na channel was important for the dominant negative effect to occur. The findings revealed a new role for β_1-subunit in cardiac voltage-gated sodium channels by promoting α-α subunit interaction (Fig. 13). This in turn may lead to a dominant negative effect when one of the α-subunits shows a mutation leading to defective trafficking (Mercier et al., 2012).

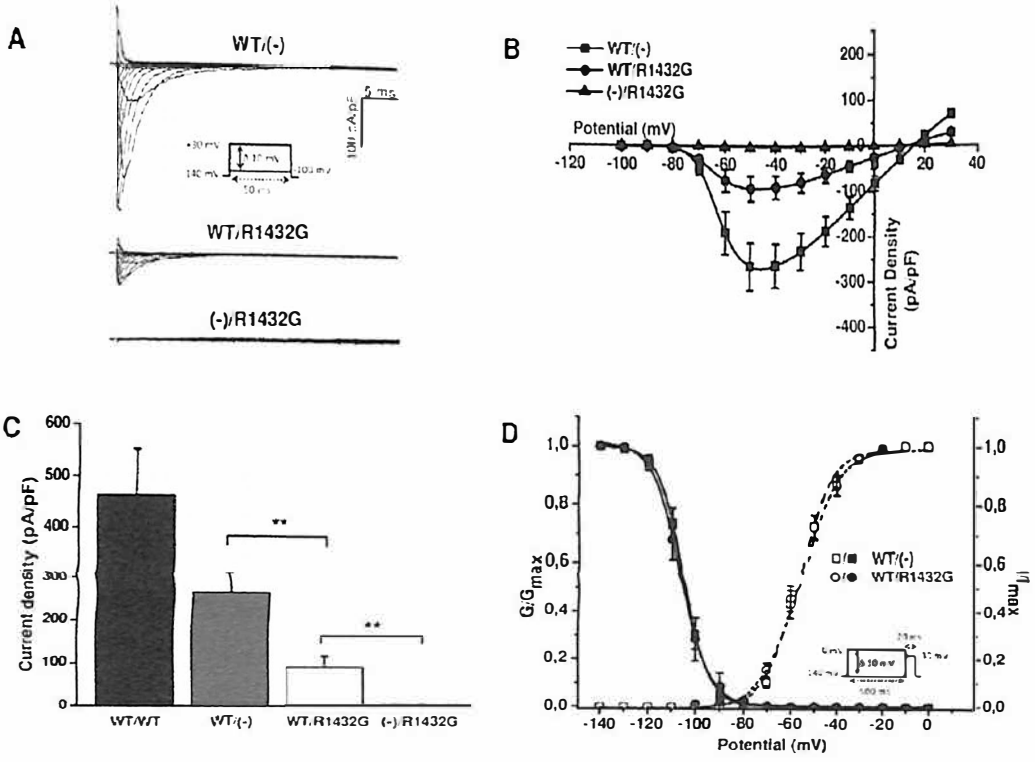

Figure 13: In the presence of mutant sodium β1 subunit, R1432G, the sodium current density of wildtype channels was reduced. (A-B), Whole cell voltage clamp recordings of cells containing wildtype, wildtype/R1432G, and R1432G alone. **(C),** when expressed with wildtype, the mutant caused a decrease in sodium current density. **(D),** Dominant negative does not affect voltage-related activation/inactivation of channels) (Mercier et al., 2012).

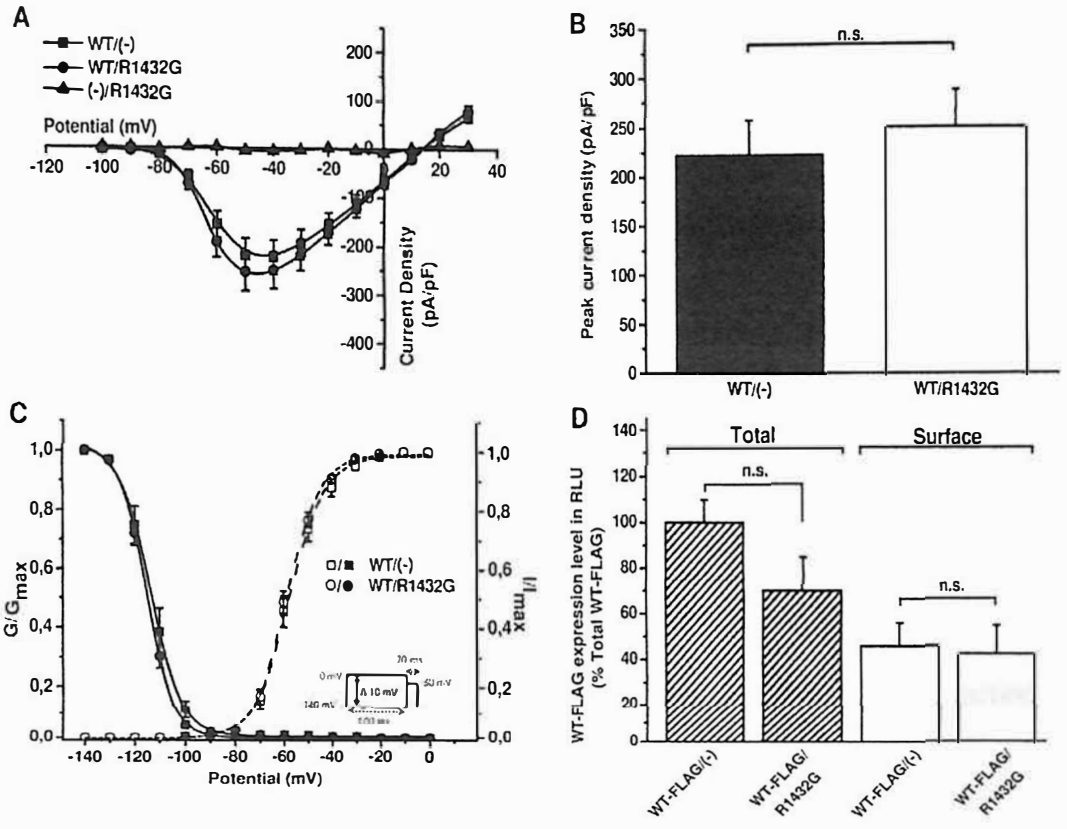

Figure 14: In the absence of β_1, R1432G dominant negative effects are abolished. Opposite results from previous experiment. Mutant protein did not induce dominant negative activity on wildtype (Mercier et al., 2012).

5.4: Cancer

Dominant negative mutations are also an important tool to investigate the mechanisms of cell proliferation and cancer. In the field of cancer research, dominant negatives play an important role because it is believed that tumor suppressor genes such as p53 fail as a response because they themselves possess inhibitory dominant negative effects. In order to investigate this, the roots of cell proliferation, such as ribosomal RNA processing, were examined. In mammalian cells, for cell proliferation and ribosomal RNA,

a protein complex called the PeBoW-complex regulates processing. The complex consists of the Pes1, Bop, and WD12 proteins (Grimm et al., 2006). Ribosome biogenesis is described as a tightly regulated process that occurs in the nucleolus. Grimm et. al (2006) is a detailed investigation of this.

In this study, the researchers observed the behavior of the Pes1 protein, which is a highly conserved protein in eukaryotes and contains several domains. In this protein a highly conserved N-terminal pescadillo-like protein domain is present at the N-terminus. The term "pescadillo-like", describes a protein that plays a vital role in cell proliferation and in extreme cases, tumor growth. The BRCA1 C-terminus (BRCT) domain is also present in the Pes1 protein. This domain is responsible for "checkpoint" functions in response to DNA (Haque et. al, 2000). The protein also possesses 3 nuclear localization sequences distributed over the protein, which function by tagging proteins for import to the cell nucleus (Haque et. al, 2000). Dominant negative mutants were constructed by inducing deletions in these areas. The mutants were expressed in the TGR-1 cell line of rat fibroblasts. Expression levels where determined by using western blot analysis. Numbering "M1-M8" identified these mutants and the area of the mutant protein that was deleted determined this. For example, mutants that possessed a 54 N-terminal amino acid deletion were in M1. Indirect immunofluorescence assays were done to determine the presence of these proteins at the nucleolus. They observed that in the N-terminal (M1) and

C-terminal (M5) truncated mutants; potently inhibited cell proliferation and triggered reversible cell cycle arrest (Figure 15).

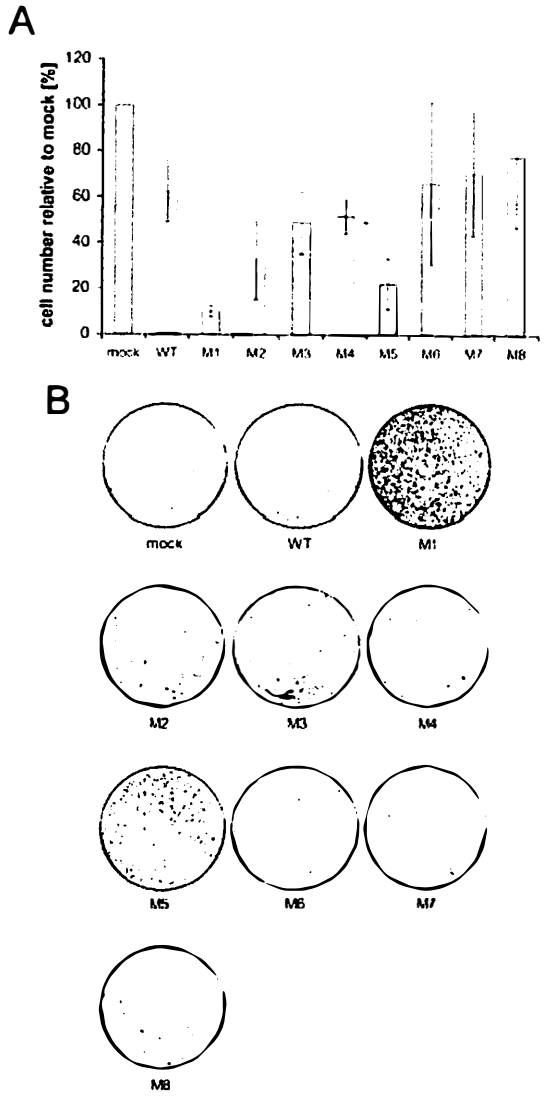

Figure 15: Dominant negative Pes1 M1 and M5 mutants inhibit cell proliferation. (A), Histogram shows stably transfected cells with the indicated constructs. (B), M1 and M5 overexpression caused cell cycle arrest shown by inhibition of cell proliferation as compared to the other cell groups (Grimm et al., 2006).

The latter part of the result was observed through exposing the cells to a BrdU light assay to test for photosensitivity, which halted cell proliferation (see Fig. 15). These two groups of mutant cells also inhibited pre-rRNA processing. In these experiments they concluded that the dominant effect of M1 and M5 mutants is mediated by the of PeBoW-complex (Grimm et al., 2006).

In 2001, Li and Koromilas studied the activity of double-stranded RNA (dsRNA)-activated protein kinase (protein kinase dsRNA-dependent) and its role in the regulation of protein synthesis by phosphorylating the α-subunit of eukaryotic initiation factor 2(EIF2) (Li & Koromilas, 2001). EIF2 is a vital factor for protein synthesis that forms a ternary complex with GTP, and the initiator methionine-tRNA (Clemens, M. J., and Elia, 1997). So essentially, EIF2 is involved with aiding in the transduction of signals. It is because of this, they asked the question of whether PKR has a role in mediating the antiviral and antiproliferative actions of interferons. Interferons are proteins that allow communication between cells to trigger the protective defenses of the immune system (Alberts et. al, 2008). In order to answer this question, Li & Koromilas generated an alternatively spliced form of PKR with a deletion of exon 7 (PKRΔE7). This truncated protein exhibited dominant negative function by inhibiting both PKR autophosphorylation and EIF2 α-unit autophosphorylation (Li & Koromilas, 2001).

In order to clone the mutant PKR, they amplified and sequenced the PKRΔE7 cDNA from human leukemia Jurkat T cells. Viral transfection in HeLa S3 cells enabled the expression of PKRΔE7 bearing a FLAG epitope. Immunoprecipition assays through western blot analysis used an anti-FLAG antibody to detect PKRΔE7. In their data,

PKRΔE7 exhibits a dominant negative function. They observed this by measuring the activation of endogenous PKR by autophoshorylation in the protein extracts by immunoprecipitation. With the PKE□E7 mutants there was an inhibition of autophosphorylation as shown by the change in the ratio of phosphor-PKR to total PKR with the mutant (Figure 16). Co-expression of wildtype PKR and the mutant also inhibited activation of the EIF2 alpha subunit (Li & Koromilas, 2001).

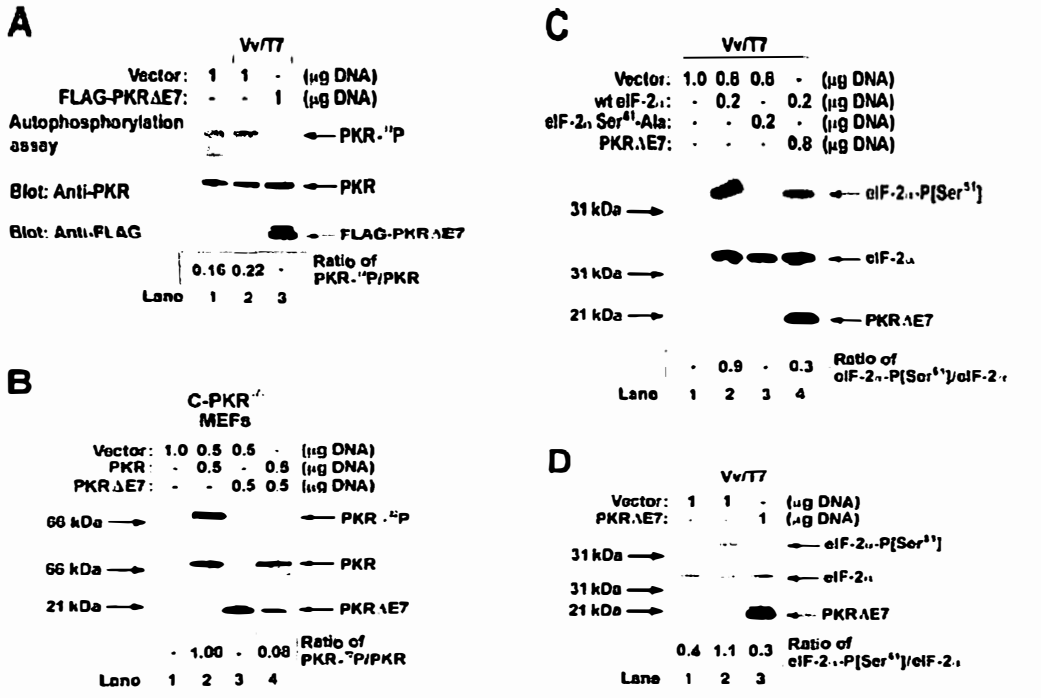

Figure 16: Western blot assay of dominant negative function of PKRΔE7. (A), HeLa cells transfected with LipofectAMINE with Vector DNA, WT human PKR cDNA, PKR Δ E7, WT human cDNA and PKR Δ E7. Western blot of phosphor-PKR, total PKR and the flag-tagged mutant PKRΔE7 **(B)** C-PKR MEFs, fibroblasts transfected with vector DNA, wildtype PKR cDNA, and mutant PKRΔE7 c-DNA. **C)** Cells transfected with wildtype eif2 (wt-eif-2) or a non-phosphorylatable eif-2 (eif-2-Ser-Ala). The mutant PKRΔE7 is less able to phosphorylate eif2. **(D)** transfection with vector or PKRΔE7 inhibits phosphorylation of eif2 as shown by the ratio of phosphorylated to un phosphorylated protein (Li & Koromilas, 2001).

They also tested the functionality of PKRΔE7 in yeast cells at lower levels do to the toxic effect on the cells. In lower levels, PKR can substitute for the function of GCN2, which is the primary EIF-2 kinase in yeast. It seems that this experiment was just used as verification that PKR and the mutant could be expressed in yeast. This was confirmed by using western blot analysis. They further verified the dominant negative function of PKRΔE7 by using reporter assays in HeLa cells and mouse fibroblasts from PKR knockout mice. The PKRΔE7 cDNA was transfected in the presence or absence of the wildtype cDNA (Fig. 17). When co-expressed with the wildtype, the mutant PKRΔE7 activates the reporter gene β-galactosidase protein, as opposed to it being inhibited when the wildtype PKR is expressed alone (Li & Koromilas, 2001).

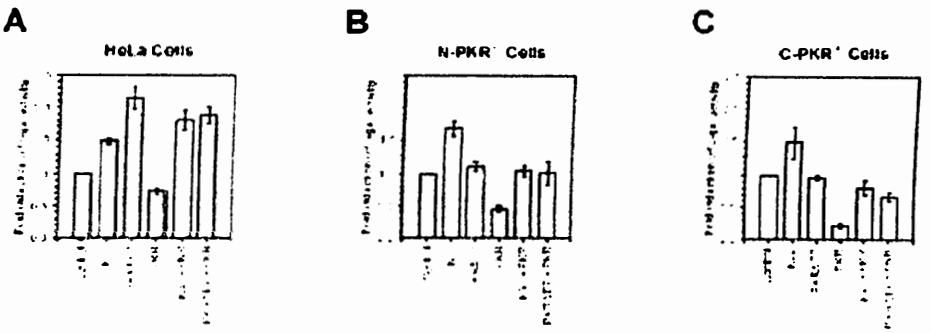

Figure 17: Induction of gene expression by PKRΔE7. (A), immortalized fibroblasts derived from mice with target deletions in exons 2 & 12 (B). (C), cells transfected with beta-galactosidase in the presence of inhibitor K3L DNA (Li & Koromilas, 2001)

In these experiments, they have shown that PKRΔE7 exhibits dominant-negative function in PKR activation in human, mouse, and yeast cells. This is through the inhibition of autophoshorylation and phosphorylation of EIF2 (Li & Koromilas, 2001). The importance of these results is that when this occurs, this inhibits the proliferation in cancer cells thus inhibiting tumor growth. Further investigation of the pathways of PKR and regulation could lead to broader understanding in signaling pathways and cell proliferation, which could aid in the development of different approaches to treating cancer.

Another cancer study focusing on androgen receptors and prostate cancer was based on the logic that if a dominant negative form of an androgen receptor with a deletion of the NH_2- terminal transactivation domain is dimerized with a full-length receptor, this would inhibit the transcriptional activity (Edwards, Krishna, Grigor, & Bartlett, 2003). In researching prostate cancer, dominant negative strategies are used to aid in finding more effective treatments. Since prostate cancer is the second leading cause of cancer related deaths in American men, effective treatment for this cancer would improve the lives of large numbers of people. A treatment that relies on androgen deprivation has been used as a treatment to shrink tumors and keep them from reoccurring.

Titus reported in a 2012 publication that despite the reduced levels of androgen due to the therapy, the cancer would reoccur due to the incomplete clearance of cancer cells. Therefore, study of the androgen receptor and it ligands has been connected to the so-called castration-recurrent prostate cancer growth. Titus et al (2012) used a ligand-dependent dominant-negative mutant androgen receptor ARΔTR expressed in CWR-R1 tumor mouse models. In this mutant, they deleted the NH_2 terminal amino acid residues of the protein

and infected the CWR-R1 cells with a lentivirus carrying the cDNA of the mutant gene combined with a reporter gene with a luciferase tag. This works because they deleted the ligand-binding site of the receptor. They used western blot analysis to assay for the expression of the protein.

The expression of this mutant protein decreased the growth of CWR-R1 tumors in the presence and absence of exogenous testosterone. They assessed tumor growth by injecting ARΔTR-transduced cells into nude mice to generate CWR-R1 tumors and the growth was observed over a course of eight days. They noticed a reduction in growth of tumor cells due to reduced signal impaired by ARΔTR (Fig. 18). These findings elucidate that for castration-recurrent prostate tumor growth to occur, normal function androgen receptor signaling by androgen receptor ligands is required (Titus et al., 2012). The inhibition of the androgen ligand binding would disable the production of androgens necessary for the growth of the CWR-R1 tumors. It is then theorized that targeting the synthesis of testosterone and dihydrotestosterone would inhibit activation of the receptors thus slowing the growth of tumors. Therefore, with the inhibition of androgen ligand binding, this would slow down the growth of CWR-R1 tumors. With further research on this topic, more effective treatments for prostate cancer can be discovered to battle this disease.

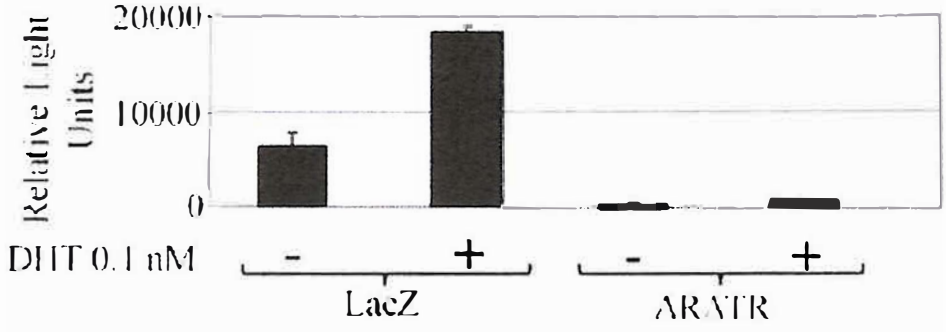

Figure 18: ARΔTR inhibits AR transactivation and CWR-R1 cell proliferation. CWR-R1 cells were transfected with MMTV-luciferase reported and assayed for luciferase activity in the presence of DHT. LacZ transgene cell displayed high luciferase activity in absence of DHT. Mutants showed reduced activity in presence or absence of DHT (Titus et al., 2012).

Chapter 6: Discussion

The premise of this paper was to investigate and gain a better appreciation of the innovative technique of using dominant negative mutant proteins to understand how they contribute to advancing biomedical sciences. As stated earlier, the discovery of the structure of DNA and the work of the human genome project provided the framework of the to unlocking the mysteries of many medical problems. Despite the large contributions, they only provided the blueprints and directions to where to truly begin. The human genome project provided an accurate and concise estimate of the amount of genes present within a human being, however, what do those genes do? The advances in cloning and DNA amplification provided the tools to answer these questions. Therefore it was up to scientific ingenuity to develop various methods that would help usher a revolution in the field of biology.

The most recent work with this technique involves investigations of agammaglobulinemia, a hereditary disorder where a person has low levels of immunoglobins leaving them prone to infections (Boisson et al., 2013). Recently it has been revealed that a dominant negative mutation in the transcription factor E47, which is responsible for the maintenance of B cells, has a role in this disorder (Boisson et al., 2013). Another study suggests that dominant negatives may have a role of being a therapeutic strategy for colorectal cancer. In this study, the role of activator protein 1 (AP-1), a transcription factor involved in activation of oncogene signals, is investigated.

A dominant negative mutant of c-Jun lacking the transactivation, transcription factor that works in association with AP-1, was constructed. It was then observed that when expressed with the wildtype c-Jun cell proliferation was reduced (Suto et al., 2004).

Dominant negative strategies can also aid in the understanding of how other diseases such as acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) work. Also, how we can use this knowledge to attack this particular problem and antiviral approaches in general. Currently, research with HIV and dominant negative of Vif, and accessory protein of HIV, is being studied. Vif suppresses the antiviral activity of APOBEC3G (A3G), a cytidine deaminase that inhibits retroviral replication. In this study, a dominant negative construct of Vif was made to interact with the wildtype, inhibiting this action of suppressing A3G thus leading to possible novel antiviral methods (Walker et al., 2010).

However the future of this technique lies in refining its usage in the form of utilizing a conditional knockout system, specifically the tetracycline controlled transcriptional activation system (tet-on/off system). Gossen and Bujard in 1992 were studying the control elements of the tetracycline resistance operon in Tn10 in *E. Coli.* By fusing the *tet* repressor with the activating domain of the virion protein 16 of herpes simplex virus, they noticed an activation of expression in the presence of tetracylcline and the silencing of gene expression in its absence (Gossen & Bujard, 1992). This can be applied to studies using dominant negative strategies in which the *tet* repressor is fused to the mutant cDNA coding for genes carrying a dominant negative mutation. An example could be using this technique in the KR-IGF-IR study with IGF-IR in the diabetes type II study. The mutant cDNA can

be fused with the *tet* repressor and under the conditions in which the animal is fed traces of doxycycline, which activates the tet response element, the mutant gene will express the mutant proteins and the dominant negative effects could be observed. When the doxycycline is removed from the animals diet, the gene with be silenced and the effects reversed. Such a conditional expression system could allow for the study of developmentally important genes by allowing normal gene expression during development. The technique would also result in more convincing evidence that a specific protein plays a specific role by reducing the potential for pleiotropic effects of the knock-down so that the transgenic model would have the observable traits under the knock-down condition.

Despite the usefulness of the dominant negative strategy in understanding protein function, this technique relies heavily on extensive prior knowledge of the protein being studied and the pathway it participates. This technique is limited by the current knowledge available. In the case of the G-protein, the structure of that protein has long been established along with the pathway being mapped out, thus this gave researchers the opportunity to further probe to elucidate the function characterization of the G-protein. Therefore, in order for this technique to work, the pathways must be mapped, the role of the protein of interest must be disclosed, and the structure of the protein must be understood. That is why the knockout system is still prevalent in current research. However, over time as our knowledge of more genes and pathways gains momentum in the field this can be one of the top methods for understanding a protein, its actions and interactions in its entirety.

Dominant negative strategies allow for a researcher to unravel the mysteries of how a given protein works by using a process of elimination. This process allows for the protein to be observed in defunct nature in order to understand the function and its affect in interacting with other proteins. This purpose of this paper was to discuss the overall significance of dominant negative strategies and their contribution to the research findings in the past and currently. Also, an appreciation is to be gained for this innovative and ingenious technique. The examples of their uses in these elegant experiments were designed to give a perspective of the meticulous and captivating applications of these techniques.

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EDUCATION

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Master's of Arts (Successful Defense) , Biology
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Thesis Title: The importance of studies using the Dominant Negative Strategy for functional characterization of protein interactions.

University of Maryland Eastern Shore, Princess Anne, MD **Aug 2005-May 2010**
Bachelor of Science, Biology
Graduation date: May, 2010

RESEARCH EXPERIENCES

University of Minnesota, Minneapolis, MN **June-Aug 2009**
Mentor: Jonathan Gewirtz, Ph.D.
Project Title: Elucidating the Cellular Markers in the Extended Amygdala Associated with Acute Morphine-Induced Withdrawal.
Aim: The purpose of this research was to identify cellular markers in the extended amygdala that are activated during acute morphine withdrawal through the utilization of immunohistochemistry to detect c-Fos and p-CREB. This experiment served as way to learn more about the structures associated with drug addiction and a way to devise more effective methods of treatment.
Techniques Learned: Profusion, Immunohistochemistry, Microtoming

University of Maryland Eastern Shore, Princess Anne, MD **June-Aug 2008**
Mentor: Mobolaji Okulate, Ph.D.
Project Title: Study of the Larvacidal Properties of a Brown Alga *Fucus vesiculosus*
Aim: The aim of this study was to determine the toxicity *Fucus vesiculosus* extract against *Anopheles gambiae* larvae. This information will aid in the discovery of an alternative method of *Anopheles gambiae* control.
Techniques Learned: Breeding mosquitoes, preparing blood meals, basic laboratory skills such as solution preparation, dilution, and pipetting

PRESENTATIONS AT SCIENTIFIC CONFERENCES

Michael K. Brown, Anna K. Radke, Robert L. Meisel, and Jonathan C. Gewirtz. *Elucidating the Cellular Markers in the Extended Amygdala Associated with Acute Morphine-Induced Withdrawal.* Annual Biomedical Research Conference for Minority Students. Phoenix, AZ. November 5, 2009.

Michael K. Brown, Anna K. Radke, Robert L. Meisel, and Jonathan C. Gewirtz. *Elucidating the Cellular Markers in the Extended Amygdala Associated with Acute Morphine-Induced Withdrawal.* University of Maryland Baltimore County Undergraduate Research Symposium. Baltimore, MD. October 10, 2009. **1st Place Poster, Biological Sciences.**

Michael K. Brown, Eun Yim, Ghislain Mandouma, Madhumi Mitra, and Mobolaji Okulate. *Study of the Larvacidal Properties of a Brown Alga Fucus vesiculosus.* Annual Biomedical Research Conference for Minority Students. Orlando, FL. November 6, 2008.

Michael K. Brown, Eun Yim, Ghislain Mandouma, Madhumi Mitra, and Mobolaji Okulate. *Study of the Larvacidal Properties of a Brown Alga Fucus vesiculosus.* University of Maryland Baltimore County Undergraduate Research Symposium. Baltimore, MD. October 17, 2008.

HONORS & AWARDS

Minority Access to Research Careers Undergraduate Student Training Academic Research Trainee **Aug 2008-May 2010**

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